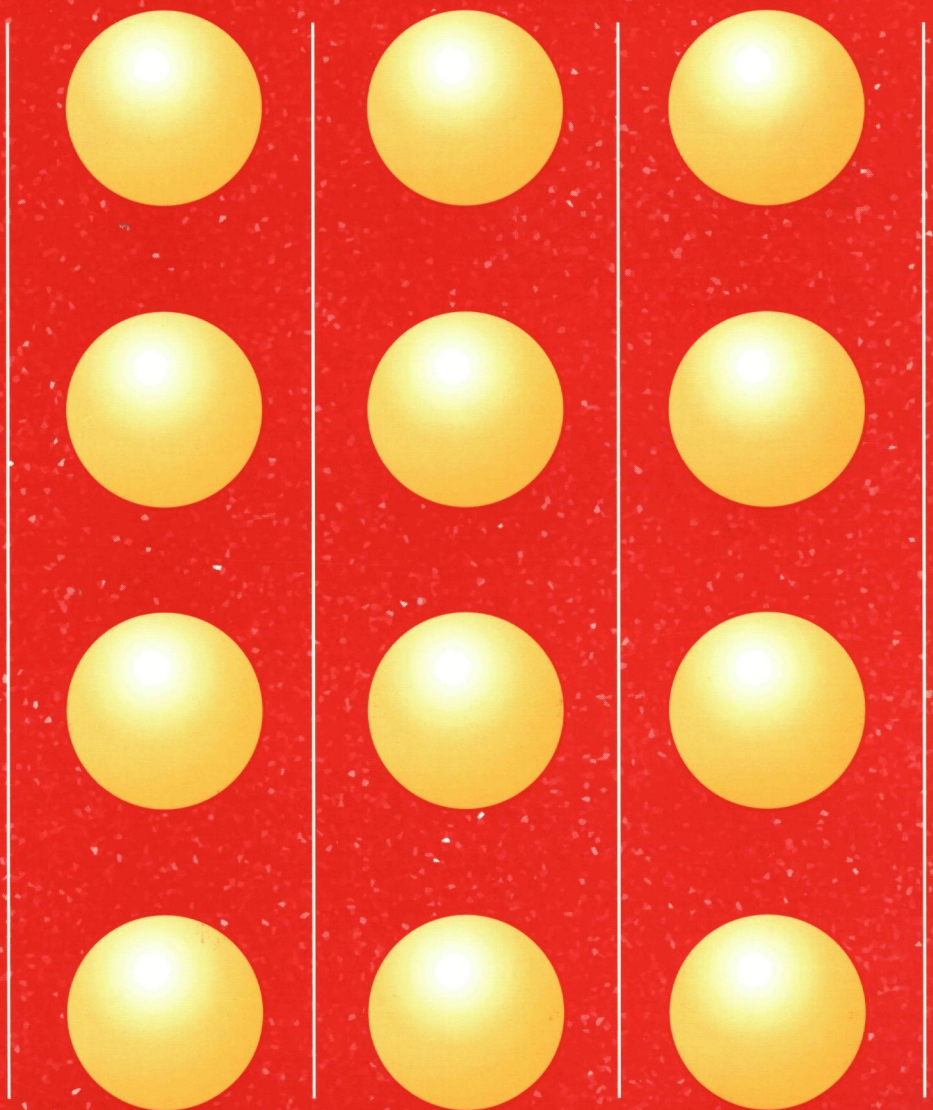
Aggressive cholesterol lowering and regression of atherosclerosis

Studies on LDL-apheresis



A.A. Kroon

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Ervaring is eenvoudig de naam die we aan de som van onze fouten geven

Oscar Wilde

Aan Désirée, Féline, Maxime
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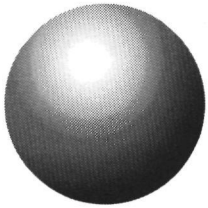

Chapter One

Introduction

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Cholesterol lowering and atherosclerosis. Clinical benefit and possible mechanisms: an update.

A.A. Kroon, A.F.H. Stalenhoef

The results of several lipid lowering randomized trials were released during the past years and have confirmed the lipid hypothesis. Reduction of cholesterol by potent drugs in clinically symptomatic or asymptomatic patients with above average cholesterol levels will substantially reduce the risk of coronary events. The present article gives a review of potent cholesterol lowering treatments and discusses developments in cholesterol lowering treatment in relation to recent primary and secondary prevention studies. Additionally, possible mechanisms of retardation of the atherosclerotic process and different outcome measures for future regression studies are summarized.

Introduction

Cardiovascular diseases are the major cause of death in the Western societies. In the Netherlands 40% of all deaths in 1993 were caused by cardiovascular diseases, of which 40% were ischemic heart disease and 23.5% cerebrovascular disease. The ability to prevent the development of atherosclerosis or, alternatively, to induce a decrease in the severity of established atherosclerotic plaques, often referred to as regression, has major implications for public health.

The validity of the lipid hypothesis has been debated for more than 40 years. The relation between total cholesterol and low density lipoprotein (LDL) cholesterol levels and the incidence of coronary artery disease (CAD) and peripheral vascular disease (PVD) is now well established [1-5]. Epidemiologic studies have shown parallel, age-related trends of atherosclerotic lesions in the abdominal aorta, carotid, and coronary arteries [6-8]. Since the beginning of the 1980s many primary and secondary prevention trials, predominantly conducted in men with hypercholesterolemia, have shown that lipid lowering regimens result in reduction of angiographic lesions and are associated with a decreased incidence of atherosclerotic events [9-42]. Most of these trials have shown slowing or arrest of progression of coronary atherosclerosis. Reports concerning femoral atherosclerosis are more scarce [12,21,23,24,29,32,33]. In several more recent clinical trials on plasma lipid regulation measurements of carotid artery intima-media thickness (IMT) have been included, since coronary and carotid atherosclerosis share coronary events as their major cause of morbidity and mortality [43-46]. Indeed, most of these studies have also shown a slowing of progression of IMT during lipid lowering treatment.

The common denominator of most of these trials is reduction of LDL cholesterol [47]. With the introduction of more potent cholesterol lowering agents, the HMG-CoA reductase inhibitors, the effects of lipid lowering on coronary and total mortality have been confirmed solidly, changing the lipid hypothesis into evidence [48]. In this introduction the background of cholesterol lowering treatment will be discussed in relation to atherosclerosis regression studies. In addition, outcome measures for lipid lowering studies are discussed, and an outline of the studies of the present thesis will be given.

Cholesterol lowering treatments

HMG-CoA reductase inhibitors

Three-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is an enzyme that catalyses the rate limiting step in the cholesterol biosynthesis [49]. HMG-CoA reductase inhibitors reduce serum cholesterol levels by inhibiting the endogenous synthesis of cholesterol, thereby upregulating LDL receptors especially in the liver

[50,51]. This leads to an increased removal from plasma of LDL as well as the precursors of LDL, intermediate density lipoproteins (IDL). This dual mechanism of decreased production and increased removal of LDL causes large reductions in serum cholesterol concentrations. Over the past few years numerous studies have shown the efficacy and safety of four HMG-CoA reductase inhibitors currently available: lovastatin, simvastatin, pravastatin, and fluvastatin. A reduction of total cholesterol ranging from 23-45% (mean 30%) can be achieved, due to a 26-42% (mean 35%) reduction of LDL-cholesterol. This effect is accompanied by a 11-32% (mean 20%) decrease in serum triglycerides and a 8-13% (mean 8%) increase of high density lipoprotein (HDL) cholesterol [52,53]. Some reports have shown an increase in lipoprotein(a) [Lp(a)] levels during treatment with these so-called statins, ranging from 8-12% [38,54].On a milligram basis simvastatin is twice as potent as pravastatin and lovastatin and four to eight times as potent as fluvastatin [55,56]. The nonapproved drug atorvastatin has been reported to decrease LDL-cholesterol by up to 60% and reduce triglyceride levels by up to 40% [57]. In general, HMG-CoA reductase inhibitors are well tolerated and side effects are neither serious nor frequent [52]. Apart from increases in liver enzyme levels greater than threefold the upper normal limit, which occur in approximately 1% of statin-treated patients, the myopathy syndrome (elevation of creatine kinase activity above tenfold the upper normal limit plus muscle pain or weakness) is the only side effect worth mentioning. The incidence of myopathy during monotherapy with statins is approximately 1 in 1000 [58], although the combination with other treatments might amplify this risk [59]. In animal studies, treatment with HMG-CoA reductase inhibitors has been shown to retard the development of atherosclerosis [50,60-62]. Moreover, until now intensive lipid lowering using HMG-CoA reductase inhibitors is the most effective in terms of inducing plaque regression and consequently reducing the number of clinical events in men with established CAD and PVD [26,27,33,36,38-42,45,46]. Recently, a prospectively planned meta-analysis that pooled data from four studies treating men and women with 10 to 40 mg per day pravastatin for 2-3 years, showed that a decrease of LDL-cholesterol of 28% from baseline was associated with 62% reduction of the combined incidence of nonfatal and fatal myocardial infarction [63]. Moreover, statins may have stabilizing and protective effects on atherosclerotic lesions, by making them less vulnerable to fissure and rupture, and by restoring the hypercholesterolemia-induced endothelial dysfunction [64,65].

LDL-apheresis

Alternative approaches of cholesterol lowering have been performed by portocaval shunting, liver transplantation and plasmapheresis [66-68]. The latter has been shown to reduce mortality of CAD [69]. New techniques have been developed to replace plasmapheresis, which remove apolipoprotein (apo) B-containing lipoproteins from plasma extracorporally. These methods make use of immuno-absorption columns,

chemical affinity columns, precipitation with heparin at low pH, and double filtration techniques [70-74]. Continuous LDL-apheresis using dextran sulfate cellulose columns selectively removes apo B-containing particles from plasma by specific affinity between dextrans and apo B [72]. The performance of regular apheresis permits the achievement of lower levels of LDL-cholesterol, which are usually not possible to attain with drug therapy alone [75-78]. Adverse events from LDL-apheresis are infrequent and similar to any extracorporeal treatment. They mainly include hypotension and chills, which have been reported with frequencies ranging between 0.2 to 1.1% and 0.3 to 6.0%, respectively [75,78]. LDL-apheresis has shown to change peripheral and cerebral hemodynamics favourably by short-term improvements of the rheological properties of whole blood and probably also by restoring the endothelium-dependent vasodilation [79,80]. The application of this method may offer opportunities in the prevention of progression, or even inducing regression of atherosclerosis as has been shown in selected patients with a primary hyperlipidemia and established CAD or PVD [32,81-87].

Clinical trials

In 1994, convincing evidence for the value of lipid lowering therapy in reducing clinical CAD events has been published in a meta-analysis of 28 randomized controlled, primary- and secondary-prevention trials by Law et al. [88]. The analysis indicated that in men 55 to 64 years of age, a 10% decrease in total cholesterol produced a 7% reduction in ischemic events during the first 2 years of the trial, a 22% reduction during years 2 to 5, and a 25% reduction after 5 years of the trial. These findings are consistent with a lag period between the initiation of therapy and a reduction in clinical events.

Primary prevention trials

The early primary prevention trials were too small to demonstrate significant changes in mortality [89]. The Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT), a 10-years placebo-controlled study in 3806 men with elevated cholesterol [14,15], and the 5-years Helsinki Heart Study (HHS), in 4081 men with elevated LDL and/or very low density lipoprotein (VLDL) cholesterol [19], both showed a reduction in coronary events after long-term treatment with cholestyramine and gemfibrozil, respectively (Table 1). The investigators of the LRC-CPPT showed that each 1% reduction in serum cholesterol would result in an approximately 2% reduction in the risk of CAD [15]. After the disappointing results on mortality of the very large World Health Organization Clofibrate Trial [9], the LRC-CPPT and the HHS actually initiated the wide acceptance of the favourable effects of lipid lowering treatment on cardiac mortality or CAD incidence, although much debate was raised by the increase in non-

Table 1. Primary prevention studies

Study [ref.no.]	number of patients	mean study duration (yr)	total cholesterol	non-fatal MI	all CAD events	non-CAD mortality
LA-VA [89]	846	7	-13%	-32%	-23%	n.c.
WHO-clofibrate [9]	10627	5.3	-9%	-26%*	-20%*	+36%*
LRC-CPPT [14]	3806	7.4	-9%	-19%	-19%*	+33%
HHS [19]	4081	5	-10%	-36%	-34%*	+25%
WOS-COPS [42]	6595	4.9	-20%	-31%*	-31%*	-11%

*Data represent percent changes of the intervention group versus the control group. MI, myocardial infarction; CAD, coronary artery disease; n.c., not changed; *, statistically significant change*

cardiac mortality (Table 1). This excess of deaths in the intervention groups has been explained by the use of fibrates, but also by an overestimation due to intention-to-treat analyses and not adjusting for the regression dilution bias and the surrogate dilution effect [90-92]. To overcome some of the limitations of the LRC-CPPT, in which the decrease in cholesterol was less than expected due to poor compliance to the study-drug, or of the HHS, which used an agent less effective in lowering cholesterol, the West of Scotland Coronary Prevention Study (WOSCOPS) was designed using one of the HMG-CoA reductase inhibitors (Table 1) [42]. In this double-blind, randomized, placebo-controlled trial 6,595 men between 45 and 64 years of age with a mean LDL-cholesterol of 5.0 mmol/L were treated with 40 mg per day pravastatin or placebo for a mean of 4.9 years. LDL-cholesterol was lowered on the average by 26% and the major endpoint, death from CAD or nonfatal myocardial infarction showed a significant relative reduction of 31%, whereas overall mortality was reduced by 22% without excess of noncardiovascular deaths. This study unequivocally showed that primary prevention with a well tolerated and potent drug, in a population at relatively low risk for CAD and no history of myocardial infarction, is effective and may also have major impact for healthcare costs. The clinical benefits of lipid-regulating therapy in primary prevention trials forgoing WOSCOPS have been less dramatic and a matter of constant debate. However, hypercholesterolemic individuals without established CAD represent an important population who will eventually die or become disabled by a coronary event. Several studies are underway, which may provide more information on the bene-

fits and risks of several treatment modalities in the primary prevention of CAD [93].

Secondary prevention trials

The Scandinavian Simvastatin Survival Study (4S) provided definitive evidence of the benefit of lowering LDL-cholesterol in patients with established CAD [39]. In this double-blind, randomized, placebo-controlled study, 4444 men and women with serum total cholesterol levels between 5.5 and 8.0 mmol/L were allocated to 20 to 40 mg per day simvastatin or placebo. After a median treatment period of 5.4 years, LDL-cholesterol was lowered by on the average 35%, and the total mortality was reduced by 30% because of a CAD mortality rate reduction of 42% in the simvastatin-treated patients. Evidently, lipid lowering in a population at high risk of coronary events is even more effective in reducing coronary and total mortality. Since the separation of cholesterol lowering trials into those studying primary and those studying secondary prevention has little foundation in coronary pathology, WOSCOPS and 4S indicate that more aggressive lipid lowering further reduces the progression of CAD. Indeed, renewed meta-analysis of randomized cholesterol lowering trials has shown a regression line between CAD and total mortality versus the percent of cholesterol reduction, indicating that further lowering of total cholesterol concentrations is associated with a decreased mortality [90]. Only recently, the Familial Hypercholesterolaemia Regression Study [87], the first randomized, controlled study using LDL-apheresis was published. Biweekly LDL-apheresis plus 40 mg per day simvastatin aggressively decreased LDL-cholesterol levels by 53%, which was significantly different from the 44% reduction induced by 40 mg/day simvastatin plus 20 g per day colestipol or 16 g per day cholestyramine. Despite these differences in LDL-cholesterol reduction, the mean percent diameter stenosis of the coronary arteries and the number of patients classified as progressors and regressors after 2 years of treatment did not differ significantly between the two groups. Although LDL-cholesterol was further lowered than in any of the above mentioned studies, the differences between the two treatment groups may have been too small and the intervention period too short to confirm the assumption that more aggressive lowering of LDL-cholesterol induced more reduction in CAD. As has been indicated by the meta-analysis of Law et al. [88], the greatest changes in atherosclerosis may be found after more than 2 years of intervention. Moreover, in a recent analysis of 11 angiographic coronary atherosclerosis regression studies it was plotted that in patients with CAD, reduction of LDL-cholesterol by 44% for the duration of 2.6 years should completely arrest the progression of the disease [94].

The extent of cholesterol lowering

Current international guidelines for the secondary prevention of CAD in Europe and

the USA stipulate that LDL-cholesterol levels should be reduced to 3 to 3.5 mmol/L and to 2.6 mmol/L or below, respectively [95,96]. However, the recently published subgroup analysis of the 4S Study showed that percentage reductions in LDL-cholesterol (32-37%) and decrease in relative risk of CAD (32-36%) were comparable and constant across all quartiles of baseline LDL-cholesterol, which ranged from below 4.39 mmol/L in the lowest quartile to more than 5.39 mmol/L in the highest quartile [97]. This indicates that, at least in subjects with total cholesterol between 5.5 and 8.0 mmol/L, the percentage reduction in LDL-cholesterol rather than its absolute level on treatment is determinant of clinical benefit, since subjects in the 4S study with LDL-cholesterol levels well above the recommended value showed the same reduction in CAD risk as those below these levels. Indeed, in the aforementioned analysis of 11 coronary atherosclerosis regression studies it has been shown that percent change in diameter stenosis of coronary arteries was correlated with percent change in LDL-cholesterol during the study and not with on-trial LDL-cholesterol concentrations [94]. These results, however, do not fit with the results of Harvard Atherosclerosis Reversibility Project (HARP), which studied the effect of intensive lipid lowering in 40 normocholesterolemic patients [34]. After 2.5 years of treatment with pravastatin and/or nicotinic acid and/or cholestyramine and/or gemfibrozil, to lower LDL-cholesterol, increase HDL-cholesterol, and decrease serum triglycerides, no differences in coronary atherosclerosis were observed between the controls (LDL-cholesterol 3.59 mmol/L) and the actively treated subjects (LDL-cholesterol 2.23 mmol/L) as measured by quantitative coronary angiography. These results were noted in the face of significant differences between the active and placebo groups: LDL -cholesterol -41%, HDL-cholesterol +13%, and triglycerides -26%. Although, HARP was a small study and the only negative angiographic regression study, these observations may reflect the importance of other risk factors in promoting progression of CAD. Consequently the issue of cholesterol lowering to recommended, fixed target values versus guidelines in percent reduction of baseline cholesterol levels is not solved yet and needs more studies.

Mechanisms of cholesterol lowering in retardation of atherosclerosis

Several mechanisms have been suggested by which lowering of LDL-cholesterol may confer clinical benefit, including stabilization of rupture-prone atherosclerotic lesions, decreased LDL-oxidizability, improvement of vascular endothelial function, and reduction of an inflammatory or immunologic response associated with atherosclerosis [98].

Plaque stabilization

The culprit lesion causing thrombosis of the coronary arteries is thought to be a

30-50% stenosis in the majority of cases. These early lesions, characterized by a lipid-rich core, a thin fibrous caps, few smooth muscle cells and fibroblasts, and a large number of foam cells particularly at the shoulders of the caps, are prone to rupture and lead to thrombotic occlusions and consequent clinical events [64,99]. Moreover, enhanced vasoconstriction potentially promotes plaque fissuring and ulceration, which may lead to thrombosis [100,101]. Evidently, there seems to be a dissociation between the results of angiographic studies and clinical outcome: lipid-lowering therapy has shown to reduce the incidence of cardiovascular events without marked changes in coronary anatomy [102-104]. Therefore, it is most likely that treatment of hypercholesterolemia depletes the lipid core of early atheromatous plaques, and consequently stabilizes these lipid-rich lesions and prevents further complications such as intramural haemorrhage and intraluminal thrombosis, an assumption that has been demonstrated in the Canadian Coronary Atherosclerosis Intervention Trial (CCAIT) and the Stanford Coronary Risk Intervention Project (SCRIP) [36,104-106].

LDL-oxidizability

Evidence exists to suggest that oxidative modification of LDL plays a key role in the development of atherosclerosis. It has been shown that LDL must undergo oxidative modification before it can be taken up by macrophages [98,107]. Oxidative modification of LDL, which may be mediated by cell-surface receptors [108], converts it to a form recognized by the macrophage scavenger receptor [109], leading to its unlimited uptake and the formation of foam cells, an early hallmark of atherosclerotic plaques. In addition to promoting excess lipid accumulation, oxidized LDL may facilitate the progression of atherosclerotic lesion by disrupting the normal endothelial cell function [110]. The finding of oxidized LDL and lipid peroxides in areas of atherosclerotic plaques and the observations that antioxidant treatment with probucol and vitamin E slows the progression of atherosclerosis in the animal model strongly support the role of LDL modification in atherogenesis [111-115]. However, a decrease in oxidative susceptibility alone is not sufficient to attenuate atherogenesis when cholesterol levels remain markedly elevated [116,117]. Recent evidence indicates that oxidized LDL inhibits endothelium-dependent arterial relaxation by diminishing the release of vasoactive compounds such as endothelium-derived relaxing factor [118]. Moreover, in a case-control study of 270 subjects in the Helsinki Heart Study it has been shown that mean levels of antibodies against oxidized LDL were significantly higher in individuals who had a coronary fatal or nonfatal event compared to controls [119].

Endothelial cell dysfunction

The normal endothelium participates in the regulation of the vascular tone by releasing substances with both anti-aggregating and vasodilating properties, such as

prostacyclin and endothelium-derived relaxing factor (EDRF), identified as nitric oxide (NO), and also substances with contracting properties, such as endothelin [120,121]. Early in the process of development of atherosclerosis increased reactions to contractile agonists and attenuated vasodilating responses have been observed [122]. It has been shown in animal models that the release of protective, anti-aggregating and vasodilating substances is diminished in atherosclerotic vessels and also as a consequence of hypercholesterolemia per se [123-129]. In humans, coronary atherosclerosis and hypercholesterolemia have also been associated with dysfunction of endothelium mediated vasomotion, which impairs coronary or myocardial blood flow and forearm blood flow [130-134]. Lipid lowering therapy has shown to improve the endothelium mediated vasomotion after relative short periods of time [135,136]. For example, Egashira et al. [135] demonstrated a significant improvement in coronary blood flow in response to acetylcholine infusion in patients with hypercholesterolemia and pravastatin therapy. Only recently, Anderson et al. [137] showed that the addition of antioxidant therapy with probucol to cholesterol lowering treatment with lovastatin improved acetylcholine-induced endothelium-dependent vasodilation significantly more than cholesterol lowering alone. Moreover, in this process of reversal it has been found that functional improvements of the coronary circulation precede the structural, anatomical improvements [138].

Reduction of inflammatory or immunologic response

The initial inciting event in atherosclerosis is intimal lipid deposition, which is followed by recruitment of inflammatory cells (monocytes and T-lymphocytes) into the intima, smooth muscle cell accumulation, and elaboration of collagen and matrix proteins by smooth muscle cells. These processes have been shown to be mediated by minimally modified LDL. They induce the expression of and interplay between adhesion molecules (selectins, ICAM-1, and VCAM-1), monocyte chemotactic proteins (e.g. MCP-1), growth factors (e.g. PDGF), and cytokines (IL-1, IL-4, IL-6, TNFα, and INFγ)[110,139,140]. It has been shown that systemic administration of oxidized LDL stimulates leucocyte adhesion and platelet aggregation to the vascular endothelium [141]. In both men and rabbits, autoantibodies are found which recognize and selectively bind oxidized LDL [111]. Moreover, the titre of autoantibodies binding malondialdehyde-LDL was found to be an independent predictor of the progression of carotid atherosclerosis in man [142]. The proliferation of smooth muscle cells is promoted by a number of growth factors, including IL-1β and IL-1α, which are secreted by activated monocytes, macrophages, and endothelial cells [143]. Oxidized LDL has been shown to induce T-cell activation, which modulates the IL-1β and tumour necrosis factor (TNFα) release [144], whereas IL-4 has been found to induce 15-lipoxygenase activity of human monocytes, inducing lipid peroxidation [140]. All these data indicate that several potential atherosclerosis reversal mechanisms exist, of whom reduction of the number of LDL particles is a central one.

Outcome measures for future cholesterol lowering studies

Quantitative Coronary Angiography (QCA)

Quantitative methods for analyzing the extent of coronary atherosclerosis from arteriograms have been developed and extensively evaluated [145-148]. In lipid lowering trials, hard clinical endpoints such as improved survival and reduction of major cardiac events are generally accepted outcome measures, however, they require numerous patients over a long intervention period [14,19,23,39,42]. At the moment, QCA is the best surrogate endpoint for evaluation of lipid-lowering therapy in CAD [25-27,30,33-36,38,40,41] and has been shown to correlate strongly with future coronary events [149,150]. The application of QCA, however, has several limitations such as the diffuse character of coronary atherosclerosis, the complexity of stenosis geometry, the foreshortening in non-orthogonal views, the insufficient contrast staining and the limited resolution of the radiographic chain [151,152]. Moreover, there seems to be a dissociation between the results of angiographic studies and clinical outcome: lipid-lowering therapy has shown to reduce the incidence of cardiovascular events without marked changes in coronary anatomy [153]. However, as discussed before, treatment of hypercholesterolemia has shown to stabilize minor atheromatous plaques by depleting the lipid core. Therefore, not the relatively small changes in angiographic lumen size, but plaque stabilization appears to decrease the risk of disruption and consequently the clinical events [104]. Given these small changes in the severity of lesions and the unclear clinical benefits of such changes, the addition of measurements that predict the functional significance of alterations in coronary stenosis seems important [154].

Assessment of regional myocardial perfusion

The cumulative effects of all anatomical and functional abnormalities of the epicardial coronary arteries, in combination with possible dysfunction of conduit and resistance vessels, are responsible for the ultimate effect of atherosclerosis on myocardial blood flow. The regional myocardial blood flow assessed by digital subtraction angiography and followed by videodensitometric calculation of the hyperemic mean transit time (HMTT) of contrast has been shown to be an important functional measure for the hemodynamic consequences of vascular stenosis [155-157]. Moreover, it reflects the integrated flow capacity of the entire coronary arterial and arteriolar vascular system, which has also been shown by Gould et al. [158], using the noninvasive assessment of the myocardial perfusion by rest dipyridamole positron emission tomography. In one of the previously cited angiographic studies it was suggested that increased coronary flow induced by enhanced vasodilation after lipid lowering therapy may also involve the smaller resistance arteries of the myocardium [133]. This is in agreement with data after LDL-apheresis evaluating skeletal muscle and cerebral circulation after acute reduction of serum cholesterol levels [79,80], and data from positron

emission tomography [158,159]. So, in addition to the anatomical assessment of changes in the luminal encroachment by QCA, functional measurements of coronary flow should be added to evaluate the impact of lipid lowering interventions.

Non-invasive assessments of CAD or PVD

Intima-media thickness of the carotid artery

In several more recent clinical trials on plasma lipid regulation and risk factor analysis, measurements of carotid artery intima-media thickness (IMT) have been included, since coronary and carotid atherosclerosis share coronary events as their major cause of morbidity and mortality [43-46]. The measurement of the IMT of the carotid artery has been shown to be a reproducible method for the analysis of early vascular lesions [160,161]. It has also been found that high-resolution B-mode ultrasonography of the carotid artery is an effective and accurate method in the assessment of atherosclerotic changes of the arterial wall [162-164]. This technique has now been applied in a number of studies and it has been shown that the IMT of the carotid artery reflects generalized atherosclerosis, indicated by its association with coronary and lower extremity atherosclerosis [165-171].

Ankle-arm index and femoral artery Doppler spectrum analysis

Peripheral vascular lesions of the lower extremities can also be detected non-invasively using ultrasound techniques. It has been shown that these techniques are important surrogate variables for clinical end-points in quantitative measurements of hemodynamically significant atherosclerotic manifestations [172]. The ankle/arm systolic pressure ratio at rest and during hyperemia is a reproducible method to determine the presence of arterial insufficiency in the lower limbs [173,174]. To differentiate between hemodynamic significant stenotic segments located in the aortoiliac or the femorotibial tract, measurement of this pressure ratio should be combined with the assessment of blood flow velocities in the common femoral artery obtained by Doppler spectrum analyses [175,176]. The combined use of both techniques at rest and during reactive hyperemia has been shown to enhance the sensitivity of detection of peripheral vascular atherosclerotic lesions [167,177].

Outline of the present thesis

In this thesis we intended to study whether aggressive lipid lowering induces more retardation of the progression of atherosclerosis than conventional treatment. With the availability of LDL-apheresis we had the opportunity to compare the outcome of this relatively new method of lipid lowering with conventional drug therapy.

We first studied the effect of long-term cholesterol lowering with pravastatin in an animal model for homozygous familial hypercholesterolemia (FH), Watanabe heritable

hyperlipidemic rabbits, and assessed changes in coronary and peripheral atherosclerosis as well as endothelium-dependent vasodilation (chapter 2).

The main study of this thesis, the LDL-Apheresis Atherosclerosis Regression Study (LAARS), was an open, randomized, single center, prospective trial in primary hypercholesterolemic men with extensive CAD. In order to determine whether more aggressive lipid lowering with biweekly LDL-apheresis plus simvastatin exerted better an anti-atherosclerotic effect than conventional lipid lowering with simvastatin alone, 42 subjects were treated for 2 years. The design of LAARS, the methods of follow-up, and some baseline characteristics of the patients are described in chapter 3.

Treatments using selective LDL-apheresis remove large amounts of apo B-containing lipoproteins from the body in a short time. Sawtooth-like alterations in lipoprotein concentrations are one of the most striking differences between patients undergoing repetitive LDL-apheresis and patients on conventional therapy. The efficacy of the treatment depends on the pre- and posttreatment lipid levels, and on the posttreatment return of lipids in plasma. Moreover, changes in the relative cholesterol and protein content may lead to compositional and conformational changes of the LDL particle, which may result in a different susceptibility to oxidation. Therefore, the kinetics of the rebound of LDL-apheresis and apheresis-induced changes in LDL-oxidizability were analyzed and described in chapters 4 and 5.

The results of LAARS concerning the primary outcome variables, quantitative computer-assisted analyses of coronary angiograms and the videodensitometric measurement of the regional myocardial blood flow are presented in chapters 6 and 7, respectively. Assessments of PVD are described in chapters 8 and 9. Chapter 8 describes the determination of the prevalence of hemodynamically significant PVD of the lower extremities in heterozygous FH patients of the outpatient lipid clinic of the University Hospital of Nijmegen, using noninvasive methods of assessment. In addition, the analyses of the secondary outcome measures of LAARS, the ultra-sonographic determination of change in PVD in the carotid arteries and the aortotibial tract, are described in chapter 9.

Until recently, one of the main indications for LDL-apheresis was homozygous FH. Finally, in chapter 10 a case report of a female, homzygous FH patient, not a LAARS subject, who was treated with LDL-apheresis for more than 4 years, is presented as an example of the long-term effects of LDL-apheresis on coronary morbidity and mortality.

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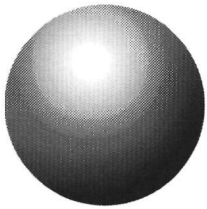

Chapter

T w o

The effect of cholesterol reduction on the endothelial function and progression of atherosclerosis in WHHL rabbits

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The effect of cholesterol reduction on the endothelial function and progression of atherosclerosis in WHHL rabbits

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In 3-month-old homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits the effect of treatment with the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor pravastatin was studied for 9 months and related to the endothelial function of the coronary arteries of isolated hearts and rings of the distal abdominal aorta. Oral administration of pravastatin in doses up to 40 mg/kg per day significantly decreased plasma cholesterol by 51% in comparison to untreated WHHL rabbits. Basal coronary flow and bradykinin-induced increase in coronary flow in Langendorff hearts of the pravastatin-treated animals were significantly greater than the flow in the control animals, whereas the metacholine-induced relaxation of abdominal aortic rings was not different and attenuated in comparison to New Zealand white rabbits. The incidence of atherosclerotic lesions in four main coronary arteries and the aorta was significantly lower in the pravastatin treated animals (25.0% and 52.8%, respectively) than in untreated WHHL rabbits (34.1% and 80.0%, respectively). The mean percentage of narrowing in the aorta was also significantly lower in the pravastatin-treated group (12.0%) than in the controls (25.2%). Significant correlations were found between the extent of atherosclerotic lesions and the bradykinin-induced increase in coronary flow versus plasma total cholesterol levels. Thus, in this model, long term cholesterol lowering treatment with pravastatin starting at an early age retards the progression of plaque formation and preserves the endothelium-dependent relaxation of the coronary arteries.

Introduction

Homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits lack the low density lipoprotein (LDL) receptor due to a monogenic disorder [1]. Therefore, normal receptor-mediated clearance of LDL in vivo is impaired and consequently the grossly elevated serum cholesterol levels lead to atherosclerosis and xanthomatosis at premature age, which progress with aging [2,3]. The atherosclerotic lesions closely resemble those in humans, and therefore this animal serves as a unique model of familial hypercholesterolemia in men [1,2].

Atherosclerosis can alter vascular reactivity [4]. The normal endothelium participates in the regulation of the vascular tone by releasing substances with both anti-aggregating and vasodilating properties, such as prostacyclin and endothelium-derived relaxing factor (EDRF), identified as nitric oxide (NO), and also substances with contracting properties, such as endothelin [5]. In the process of development of atherosclerosis, increased reactions to contractile agonists and attenuated vasodilating responses have been observed [6]. The release of protective, anti-aggregating and vasodilating substances is diminished in atherosclerotic vessels [7-9], and also as a consequence of hypercholesterolemia per se [9,10].

Evidence is emerging that oxidative modification of LDL is an important feature in the development of atherosclerosis [11]. Moreover, the accumulation of native and oxidatively modified LDL in atherosclerotic plaques [12,13] has been shown to impair endothelium-dependent vasodilation [14].

Pravastatin is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme that catalyses the rate limiting step in the cholesterol biosynthesis [15]. In animal studies, treatment with HMG-CoA reductase inhibitors has been shown to retard the development of atherosclerosis [16-19].

In the present study, the effects of cholesterol lowering by long term administration of pravastatin was studied on the progression of atherosclerosis in WHHL rabbits. The results were related to measurements of the endothelial function in both coronary arteries of the isolated heart and in the isolated distal aorta from the same animal.

Materials and Methods

Animals

Homozygous WHHL rabbits were bred by crossing and back-crossing with New Zealand white (NZW) rabbits [20]. They were housed individually in metal cages in a room maintained at constant temperature and humidity, and maintained on a regular diet (LK04, Hope Farms, Woerden, the Netherlands). Three-month-old animals

matched for sex and cholesterol, were divided into two groups. Ten rabbits were fed 0.06% (w/w) pravastatin-enriched pelleted chow at 100 g per day (20 mg/kg per day) for the first 5 months of the study and 0.12% (w/w) pravastatin (40 mg/kg per day) from the sixth month to the end of the intervention at 9 months (group P; six males and four females). A second group of ten rabbits consumed unmodified LK04 pelleted chow and served as controls for progression of atherosclerosis (group C; six males and four females). Food intake was monitored daily for all groups. Body weights were determined every 4 weeks.

Three animals from group P died at the age of 6, 7 and 9 months, in the third, fourth and sixth months of the study respectively. The cause of death shown by autopsy was a *Pasteurella multocida* infection of the lungs and the upper respiratory tract. Measurements of endothelial function and the extent of atherosclerosis are presented for 17 animals.

In order to compare the effect on endothelial function of the coronary arteries and a segment of the distal abdominal aorta, six 6-month-old NZW rabbits (three males and three females) were also studied as normal controls.

Measurements of lipid levels

After an overnight fast, blood samples were drawn from ear arteries for measurement of serum lipid levels every 4 weeks. Two series of baseline levels were taken. Plasma cholesterol and triglycerides were determined by enzymatic methods on a centrifugal analyzer using commercially available reagent for cholesterol (CHOD-PAP, no. 237574 Boehringer, Mannheim, FRG) and triglycerides (Sera-PAK, Miles, Italy, cat. no. 6639).

Determination of endothelial function

After 9 months of treatment, the rabbits were anesthetized intravenously with 6.0 mg/kg sodium pentobarbital. After heparin (1500 IU) was administrated intravenously, they were sacrificed by removing their heart and complete aorta from the ascending aorta to the ilial bifurcation. The heart was excised rapidly and put into icy saline. Retrograde perfusion of the aorta, essentially by the Langendorff method, was achieved immediately, as described in Ref. 21. Perfusion pressure was maintained constant at 60 mmHg by means of a microprocessor-controlled perfusion pump, which also calculated the coronary flow continuously. Left ventricular pressure was recorded by means of a cannula in the left ventricle. All hearts beat spontaneously. The standard perfusion medium used was a modified Tyrode solution, containing 10 mM glucose and equilibrated with 95% oxygen and 5% carbon dioxide. The temperature was kept between 37.5° and 38.0°C. A bipolar ECG and heart rate were recorded continuously. After a 15 min equilibration period using standard perfusion medium, basal coronary flow was determined. Next, a dose-response curve of bradykinin (10^{-10} - 10^{-7} M) was obtained. Seven different concentrations of bradykinin were studied in increasing sequence for each heart. After the administration of one concentration for 2 min, there

was a washout period of 5 min before the next concentration was administered. After another equilibration period of 15 min, endothelium-independent relaxation with 1 mM sodium nitrite was determined.

For measurements of isotonic displacements, a portion of the distal abdominal aorta was used. The aorta was carefully removed and placed in an oxygen/carbon dioxide enriched Krebs' solution at constant pH. During the preparation, any contact with the luminal surface was avoided to preserve endothelial integrity. Rings of 2 mm in length were mounted in 10 ml organ baths, filled with Krebs' solution at 37.5°C. The rings were given a preload weight of 3.5 g and allowed to stabilize for 90 min, during which regular washings were performed. Thereafter, they were preconstricted with 10 μM phenylephrine. Once a stable contraction level was established, endothelium-dependent relaxations were evoked by adding cumulative concentrations of metacholine (10^{-8}-10^{-4} M bath concentrations). Subsequently, rings were washed and allowed to stabilize for 45 min. After the rings were preconstricted again as described above, endothelium-independent relaxations were induced by adding cumulative concentrations of sodium nitrite (10^{-6}-10^{-2} M). In this way, nine different concentrations of metacholine and sodium nitrite were studied. Relaxations were expressed as a percentage of change of the previously established contraction with phenylephrine. All experiments with the aortic rings were performed in duplicate.

Measurement of atherosclerotic lesions

Immediately after the rabbits were sacrificed, the aortas were fixed in 10% formalin. The hearts were fixed in 10% formalin at the end of the perfusion studies. Coronary atherosclerosis was examined as described by Watanabe et al [16]. Accordingly, the heart was cut in eight equal cross-sections of ventricle. The aorta was divided into three portions: the ascending aortic arch, the descending thoracic aorta and the abdominal aorta were cut in 8, 10 and 16 - 18 equal transverse segments, respectively. These cross-sections of the hearts and the aortas were embedded in paraffin and 5 μm sections were stained with hematoxyline-eosin, Azan-Mallory and elastica-van Gieson. Luminal narrowing was assessed by computer-assisted stereologic point counting [22], using a Zeiss stereomicroscope which superimposed an integrated eyepiece graticule with 168 intersection points of horizontal and vertical lines on the transverse segments of the vessels. The internal elastic lamina was taken as the original luminal border. The percentage of luminal narrowing was calculated from the ratio of the number of points lying on and within the area bounded by the internal elastic lamina and the points within the still patent lumen. Four coronary arteries were studied: the left anterior descending artery (LAD), the left circumflex artery (LCX), the left septal artery (LSP) and the right coronary artery (RCA).

Statistical analysis

Unless indicated otherwise, a one-way analysis of variance (ANOVA) was used to

analyze differences within group means. Differences in the mean value of the body-weight, the lipid levels, the percent of narrowing of the atherosclerotic lesions in the aorta and the coronary arteries were tested for significance by Tukey's contrast method for multiple-comparison, in addition to the ANOVA. The relation of plasma total cholesterol to the extent of atherosclerotic lesions and coronary flow was quantified by using the Pearson product-moment correlation coefficient. A *P*-value of less than 0.05 was considered to be significant. Statistical analyses were performed with procedures available in the SPSS software package (SPSS Inc., Chicago, IL). Results are expressed as means ± S.D., unless otherwise indicated.

Results

General characteristics

There were no significant differences in age, sex distribution and body-weight between the comparative groups of WHHL rabbits (Table 1). During the first 6 months of the study, the pravastatin-treated group (P) and the control group (C) showed equal weight gain of about 400 g (19.1%). In the following months no increase in body weight was found, and no significant differences were present between the groups (Fig. 1).

The food consumption was both constant and comparable between the groups. The content of pravastatin after preparation of the pelleted chow before and after doubling the dosage was 60 mg/100 g and 120 mg/100 g, respectively, showing a fully active inhibition of HMG-CoA reductase in comparison to 'spiked' control chow (Dr. R. Gregg, Bristol-Myers Squibb, Princeton, NJ).

Plasma concentration of lipids

Plasma concentrations of total cholesterol at the start of the study are given in Table 1.

Table 1. Baseline characteristics of WHHL rabbits in the intervention group with pravastatin (P) and in the control group (C), and of NZW rabbits (NZW) which served as controls in the determination of the endothelial function at the end of the intervention period

Group	m/f	Age (months)	Weight (g)	Tot.chol. (mmol/l)	Triglycerides (mmol/l)
P	6/4	3.5	2183±260	12.73±3.00	3.25±0.55
C	6/4	3.5	2335±350	11.26±1.67	3.86±1.19
NZW	3/3	6.0	2700±225	1.13±0.24	0.70±0.22

Values are mean ± S.D.; m/f, number of males/females; Tot.chol., plasma total cholesterol. No significant differences are present between groups P and C

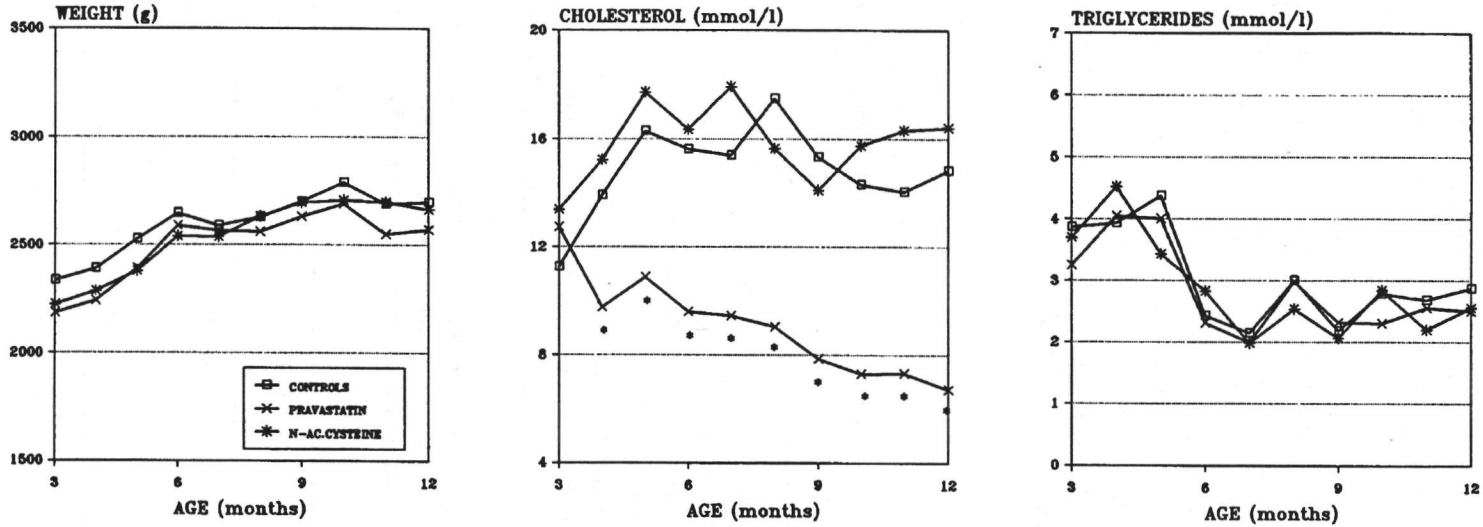

*Fig. 1. Body weight (left), total plasma cholesterol (centre) and plasma triglyceride levels (right) of WHHL rabbits in the course of treatment with pravastatin (20 and 40 mg/kg per day, at age 3-8 and 8-12 months, respectively), compared to controls. Data represent the mean at each point. No significant differences are seen between the groups for body weight and triglycerides. Differences in total cholesterol: * $P<0.001$ versus C*

In comparison to baseline levels, group C showed an increase in plasma total cholesterol of 31.6% (P <0.001). This increase mainly occurred in the first 2 months of the study (Fig. 1). Administration of 20 mg/kg per day of pravastatin caused a mean difference in plasma total cholesterol of 42.1%, in comparison to the control group. Increasing the dose to 40 mg/kg per day of pravastatin, caused a further mean difference of plasma cholesterol levels of 54.7% at 12 months (Fig. 1).

Both groups showed an identical mean change in plasma triglycerides: after the first 3 months of the study, all concentrations fell on average 36.6% (P <0.002) to a level between 2 and 3 mmol/L (Fig. 1).

The six NZW rabbits, which served as normal controls in the determination of the endothelial function, had a mean plasma total cholesterol concentration of 1.13±0.24 mmol/l and mean plasma triglycerides of 0.70±0.22 mmol/l (Table 1); their mean weight was comparable to that of the WHHL rabbits at the end of the study (Fig. 1)

Determination of endothelial function

The basal coronary flow in the hearts of the pravastatin-treated animals was significantly higher than in the controls and not significantly different from the NZW rabbits (Table 2). Coronary flow increase by bradykinin, which releases EDRF, was significantly enhanced in the hearts of the pravastatin-treated animals as compared to the controls (Fig. 2). The ratio of maximal bradykinin and sodium nitrite induced relaxation, a measure for endothelial relaxing capacity, was significantly higher in the pravastatin-treated animals than in the control hearts, and not significantly different from the ratio in the NZW rabbits (Table 2).

In contrast, no differences in the metacholine induced relaxation of the abdominal aortic rings were observed between the pravastatin-treated animals and the controls. All these relaxations were significantly lower when compared than those obtained in the

Table 2. Basal, maximal bradykinin and sodium nitrite induced coronary flow (ml/min) and the ratio of maximal bradykinin and sodium nitrite induced increase in coronary flow in WHHL rabbits (pravastatin-treated (P) and controls (C)), compared to normal NZW rabbits (NZW)

Group	*n*	Basal	Bradykinin	Nitrite	Bradykinin/ nitrite
P	7	51.2±1.9*	68.3±1.9*	67.3±2.3	1.01±0.08*
C	10	43.6±3.3	53.3±3.8	69.2±6.2	0.73±0.04
NZW	6	56.3±2.9*	69.7±2.8*	67.1±4.8	1.07±0.08*

Values are mean ± S.E.M.; n, number of rabbits in each group.
*Differences: * $P<0.05$ vs. C*

NZW rabbits (data not shown). Sodium nitrite induced relaxation of the aortic rings were comparable in all three groups.

We also studied the relationship between plasma total cholesterol levels at the end of the study and basal coronary flow, and end-of-study plasma total cholesterol levels and the maximal bradykinin induced increase in coronary flow, because the cholesterol level may be expected to be directly proportional to endothelium-dependent relaxation. The basal coronary flow showed no correlation with the plasma total cholesterol level, whereas a significant correlation was found between the bradykinin induced increase in coronary flow and the cholesterol levels in 17 WHHL rabbits (Table 3).

Extent of atherosclerotic lesions

At 12 months of age, atherosclerotic lesions of the aorta were predominantly found in the ascending and descending thoracic aorta (Table 4). In the pravastatin-treated rabbits, the mean percent of narrowing and the incidence of lesions on the cross-sections of the aorta were significantly less in all areas than in the control group.

The incidence of lesions and narrowing of coronary arteries are summarized in Table 5. Most stenoses were found in the LSP and maximal narrowing was predominantly observed at the origin of the four main coronary arteries. The incidence of all plaques was significantly lower in the pravastatin-treated rabbits than in the control rabbits. The incidence of proximal stenotic lesions at the origin of the coronary arteries was as high as 39.3% in the pravastatin-treated group and 65.0% in the control

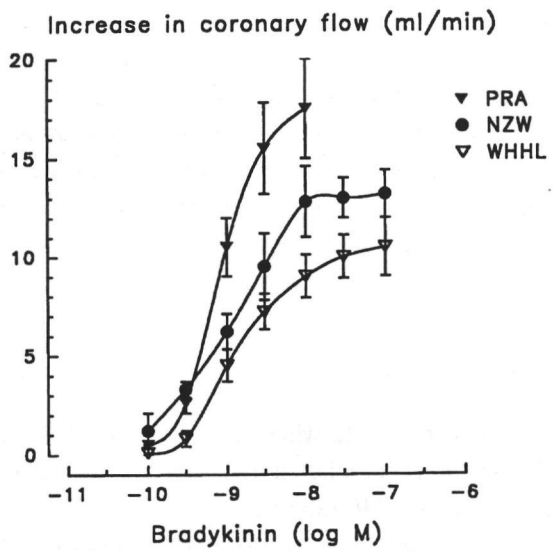

Fig. 2. The dose-response curve of bradykinin on the coronary flow of the atherosclerotic WHHL rabbits (pravastatin-treated (PRA (n=7)) and controls (WHHL (n=10)), and the normal NZW rabbits (NZW (n=6)). Data represent the mean (±S.E.M.) at each point. Differences: PRA vs. WHHL-controls, $P<0.05$

Table 3. Correlations between the plasma total cholesterol levels at the end of the study versus the basal and stimulated coronary flow, mean percentage of aortic and coronary stenosis, and incidence of the aortic and coronary stenosis in 12-month-old WHHL rabbits, pravastatin-treated ($n=7$) and untreated controls ($n=10$): the Pearson rank correlation (r) coefficients and their levels of significance (P) are given for each relation

Total cholesterol ($n=17$) versus	r	P
Coronary flow		
Basal	-0.165	0.681
Bradykinin induced increase	-0.426	0.028
Mean stenosis		
Aortic	0.626	0.002
Coronary	0.423	0.029
Incidence of stenosis		
Aortic	0.669	0.000
Coronary	0.571	0.004

group ($P < 0.05$; Pearson's chi-square test). The mean stenosis in the pravastatin-treated animals showed a tendency towards less narrowing. Correlations between plasma total cholesterol levels at the end of the study and the extent of atherosclerotic lesions in both the aorta and the coronary vessels are shown in Table 3. Significant correlations were found between the mean aortic and coronary stenosis, and the incidence of aortic and coronary stenosis versus the cholesterol level.

Discussion

The aim of this study was to elucidate whether or not cholesterol lowering treatment with pravastatin could preserve endothelial function and retard the development of atherosclerosis in homozygous WHHL rabbits.

Watanabe and Ito et al. have demonstrated that plasma total cholesterol levels in homozygous WHHL rabbits increase with maturation and decrease thereafter with increasing age during the first 12-24 months of life, whereas triglyceride levels gradually decline after the first month of life [23,24]. The initial rise in plasma total cholesterol of the control animals in the first 3 months of the present study is in agreement with these findings. No explanation was found for the drop in plasma

Table 4. Percentage of narrowing of atherosclerotic lesions (mean ± S.D. and range) and the incidence (%) of vascular lesions in the ascending thoracic, descending thoracic and abdominal aorta of 12-month-old WHHL rabbits, treated with pravastatin (P) and compared to untreated controls (C), analyzed with the point-counting method in transversal cross-sections of the aorta

Group		Ascending	Descending	Abdominal	Total
P	Mean	21.4±19.4	8.2±9.8*	6.3±6.0*	12.0±11.3*
(*n*=7)	Range	0-78.1	0-69.9	0-52.5	0-78.1
	Inc.	82.1 (46/56)¶	68.6 (48/70)¶	31.0 (39/136)¶	52.8 (133/252)¶
C	Mean	36.0±7.9	22.4±12.1	17.3±8.2	25.2±8.1
(*n*=10)	Range	2.3-81.3	0-79.7	0-75.0	0-79.7
	Inc.	100 (80/80)	98 (98/100)	61.6 (114/185)	80.0 (292/365)

Inc., incidence (the values in parentheses indicate the number of injured arteries/the number of examined transversal cross-sections); n, number of rabbits in each group
*Differences in mean stenotic lesion: *P≤0.02 versus C*
Differences in incidence (Pearson's chi-square test): ¶P≤0.0001 versus C

triglycerides of both the pravastatin-treated animals and the controls.

Homozygous WHHL rabbits develop progressive aortic atherosclerosis in 100% after the first 5 months of life. The incidence of coronary atherosclerosis gradually increases with age, from 30% in immature (5-7 months) to 55% in advanced age (25-36 months) [3,24]. Treatment with the antioxidant probucol [25,26] and the cholesterol lowering HMG-CoA reductase inhibitors [16-19] have both shown to retard the development of atherosclerotic plaque formation. The inhibitory effect on atherosclerosis of the latter seems to be dose-dependent and more pronounced in young WHHL rabbits [16,19].

Therefore, we started this study with 3-month-old WHHL rabbits and used doses of pravastatin as high as 40 mg/kg per day, causing a 51% difference in plasma total cholesterol levels to a mean of 7.29 mmol/l. Indeed, a significant retardation of the progression of aortic atherosclerosis and a reduction of the number of lesions in the coronary arteries was found in the pravastatin-treated animals, as well as a tendency towards decrease of the plaque size of the coronary atherosclerosis. The coronary lesions were mainly located at the proximal portion of the coronary vessels. This pattern of distribution of coronary lesions is similar to those previously described [24,27].

In WHHL rabbits, the plasma cholesterol level required for the development of coronary atherosclerosis is higher than the level that causes aortic atherosclerosis,

probably because of structural differences between these two arteries [3,28]. Probably, the percentage reduction of total cholesterol will determine the amount of atherosclerosis that is retarded in either the coronary arteries or the aorta. Indeed, it has been shown that 28% reduction of total cholesterol by the administration of pravastatin sodium at a dose of 50 mg/kg per day in 3-month-old WHHL rabbits suppressed the progression of coronary atherosclerosis only [16], whereas treatment with the combination of 50 mg/kg per day pravastatin and cholestyramine in the diet reduced total cholesterol by 62%, and suppressed the progression of both coronary and aortic atherosclerosis [18]. Therefore, the retardation of the progression of atherosclerosis in the aorta in our study is in accordance with these previous studies [16,18], because the plasma total cholesterol levels in the pravastatin-treated animals were 51% lower (14.90 mmol/l vs. 7.29 mmol/l). Moreover, in mature WHHL rabbits coronary atherosclerosis hardly developed when their total cholesterol levels at weaning were under 18 mmol/l [3,29], whereas baseline levels in our study ranged between 11 and 13 mmol/l (Table 1). Therefore, the incidence of coronary atherosclerosis of 34% (Table 5) in the strain of WHHL rabbits we used, is less than reported elsewhere [3,19], and may explain the less pronounced effect of pravastatin treatment on retardation of the progression of coronary atherosclerosis in this study.

It has been shown that aortic atherosclerotic preparations of WHHL rabbits have a

Table 5. Percentage of narrowing of atherosclerotic lesions (mean ± S.D. and range) and the incidence (%) of vascular lesions in coronary arteries of 12-month-old WHHL rabbits treated with pravastatin (P) and compared to untreated controls (C), analyzed with the point-counting method in 8 equal cross-sections of the ventricles

Group		LAD	LSP	LCX	RCA	All
P	Mean	7.7±9.3	13.4±14.4	10.2±14.2	6.5±5.8	9.9±5.3
$n=7$	Range	1.5-23.1	1.9-56.0	0.7-71.6	0.8-28.0	0.7-71.6
	Inc.	19.6 (11/56)*	28.6 (16/56)*	26.8 (15/56)*	25.0 (14/56)	25.0 (56/224)*
C	Mean	12.0±16.1	19.7±9.9	10.4±9.0	8.5±8.9	12.6±7.5
$n=10$	Range	0.7-50.7	1.0-78.1	0.7-80.2	1.6-56.1	0.7-80.2
	Inc.	25.0 (20/80)	47.5 (38/80)	43.8 (35/80)	20.0 (16/80)	34.1 (109/320)

*Inc., incidence (the values in parentheses indicate the number of injured arteries/the number of examined transversal cross-sections); n, number of rabbits in each group. Differences in mean stenotic lesions are not significant. Differences in incidence (Pearson's chi-square test): * $P<0.05$ versus C*

diminished endothelium-dependent relaxation in response to acetylcholine [7,8]. In addition, both in the hypercholesterolemic rabbit [9,10] and in men [30,31] it has been shown that hypercholesterolemia per se influences endothelial function and precedes the development of atherosclerosis, which may be caused by a decreased production or an increased inactivation of EDRF by native and oxidized LDL [14,32]. Only in short term studies with cholesterol-fed NZW rabbits has it been shown that administration of lovastatin, a HMG-CoA reductase inhibitor, improved the endothelial-dependent relaxation [9,10,33]. In our study, it was postulated that functional changes preceded the diminution of atherosclerosis predominantly due to cholesterol lowering. Indeed, the endothelium-dependent relaxation of the coronary arteries in the pravastatin-treated group was normal, and the bradykinin-induced increase in coronary flow was significantly correlated to the plasma total cholesterol level. Even so, 25% of the coronary cross-sections of the pravastatin-treated animals showed lesions in comparison to 34% in the control group. In contrast, a diminished endothelium-dependent relaxation of the abdominal aortic rings of the pravastatin-treated group was found, whereas these parts of the aorta showed the most pronounced preservation of normal intima (incidence of atherosclerotic lesions of 31% in the pravastatin-treated animals versus 62% in the control group (Table 4)). Yet, despite pravastatin treatment, the WHHL rabbits had plasma total cholesterol levels still well above those in normal NZW rabbits. Therefore, these results suggest local differences between the aorta and the coronary arteries in susceptibility to endothelial dysfunction by high cholesterol levels. It can not be excluded that the preservation of normal intima was more complete in the coronary arteries, but several studies have shown that the plaque as a distance barrier to the diffusion of EDRF is not primarily responsible for changes in endothelium-dependent response [10,32]. Taking account of the structural differences between the aorta and the coronary arteries, the restoration of the endothelium-dependent vasodilation in the aorta of this model may need more aggressive lipid lowering.

In conclusion, the administration of pravastatin leads to retardation of the progression of atherosclerotic plaque formation in both coronary arteries and the aorta, and to the preservation of the endothelial function of the coronary arteries, due to its lipid lowering effect. These results are encouraging with regard to lipid lowering treatment in familial hypercholesterolemia in humans.

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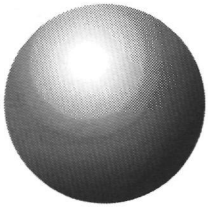

Chapter

Three

Effect of aggressive versus conventional lipid lowering treatment on coronary and peripheral atherosclerosis: design and baseline characteristics of the LDL-Apheresis Atherosclerosis Regression Study (LAARS)

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Effect of aggressive versus conventional lipid lowering treatment on coronary and peripheral atherosclerosis: design and baseline characteristics of the LDL-Apheresis Atherosclerosis Regression Study (LAARS)

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In this open, randomized, 2-years study in hypercholesterolemic men with severe coronary atherosclerosis, the effect of aggressive lipid lowering with LDL-apheresis plus simvastatin will be compared with conventional lipid lowering treatment with simvastatin alone. Treatments will be compared by means of quantitative coronary angiography and videodensitometric assessment of the coronary perfusion. Secondary, peripheral vascular disease of the carotid artery and the aorto-tibial tract will be followed by ultrasonographic techniques. Changes in endpoints will be related to changes in lipid and lipoprotein levels.

Introduction

The relation between total cholesterol and LDL-cholesterol (LDL-C) levels and the incidence of coronary artery disease (CAD) en peripheral vascular disease (PVD) is well established [1-5]. Primary and secondary prevention trials, conducted in men with hypercholesterolemia, have shown that lipid lowering regimens result in reduction of angiographic lesions and are associated with a decreased incidence of atherosclerosis [6-31]. Most of these trials show slowing or arrest of progression of coronary and femoral atherosclerosis, although studies on the latter are scarce [32]. Until now, intensive lipid lowering in men with established CAD using HMG-CoA reductase inhibitors is the most effective in terms of inducing plaque regression and consequently reducing the number of clinical events [21,22,27,29-31,33]. Quantitative methods for analysing the extent of atherosclerosis from arteriograms have been developed and extensively evaluated [34-37]. The intrinsic limitations of coronary angiography to predict physiologic effects of coronary obstruction have been well documented [38]. Therefore, assessment of the functional significance of coronary stenosis seems important. The videodensitometric assessment of the coronary perfusion has been shown to be an important functional indicator of the haemodynamic consequences of vascular stenosis [39-41].

Peripheral vascular lesions can be detected non-invasively using ultrasound techniques. The measurement of the intima-media thickness of the carotid artery has been shown to be a reproducible method for the analysis of early vascular lesions [42,43]. However, the combined use of Doppler-derived analysis of blood flow velocities in the common femoral artery and the ankle/arm systolic pressure ratio at rest and during reactive hyperemia has been shown to be a sensitive measure for the assessment of haemodynamically significant stenosis of the lower limb [44-46].
Continuous LDL-apheresis, using dextran sulfate cellulose columns, selectively removes apolipoprotein-B-containing lipoproteins from plasma [47,48]. The application of this method of hypolipidaemic treatment may offer opportunities in the prevention of progression, or even inducing regression of coronary atherosclerosis in selected patients with a primary hyperlipidaemia and established CAD [49-51].

The LDL-Apheresis Atherosclerosis Regression Study (LAARS) was designed as an open, randomized, single-centre study to determine whether aggressive lipid lowering with biweekly LDL-apheresis plus simvastatin, a potent HMG-CoA reductase inhibitor [52,53], exerts better anti-atherosclerotic effect then conventional lipid lowering with simvastatin alone or in combination with a resin, in primary hypercholesterolaemic men with extensive coronary artery disease [54]. Qualitative and quantitative computer-assisted analyses of coronary angiograms and the videodensitometric measurement of the coronary perfusion are being employed at the start of the study and after 2 years of treatment. Secondary, ultrasonographic determination of PVD in the

carotid artery and aorto-tibial tract are to be performed at 1-year treatment intervals.

From January 1990 to June 1992, 42 patients, from the Lipid and Cardiology clinics of the University Hospital of Nijmegen, were entered. In this report, we describe the design of the study, the methods of follow-up, and the baseline characteristics of the patients.

Design of the study

Men, aged between 30 and 67 years, who underwent diagnostic coronary angiography for angina pectoris were screened for eligibility. Included were patients with a mean of two successive serum total cholesterol determinations above 8.0 mmol/L or LDL cholesterol above 5.8 mmol/L, and a mean of two successive fasting serum triglyceride measurements below 5.0 mmol/L on a standard lipid lowering diet without other lipid lowering treatments, and extensive coronary atherosclerosis as shown on their coronary angiogram. Specific exclusion criteria are listed in Table 1.

To perform the prerandomization evaluations and establish eligibility, three screening visits, spaced by a month were scheduled. The aims of the study and the protocol were explained at visit 1. Instructions about a cholesterol lowering diet equivalent to the American Heart Association phase I diet were given by a dietician, and all other lipid lowering treatments were stopped. At visits 2 and 3, blood was drawn for analyses of lipids, (apo)lipoproteins, and laboratory safety measures. Exercise tests and assessments or peripheral vascular disease were performed in the meantime, as well as coronary angiography (Figure1). After visit 3, the final decision with regard to eligibility was taken and written informed consent according to the Declaration of Helsinki was

Table 1. LAARS exclusion criteria

1.	Left ventricular ejection fraction <0.35 (N=0.55-0.85)
2.	Myocardial infarction, PTCA or CABG within previous 3 months
3.	Cardiac arrhythmias
4.	Impaired hepatic function, or liver function tests with repeated values >30% above the normal range, hepatitis or biliary obstruction
5.	Impaired renal function with plasma creatinine ≥150 μmol/L
6.	Homozygous familial hypercholesterolemia
7.	Secondary hyperlipidemias due to any cause
8.	Diabetes mellitus or fasting blood glucose ≥8.0 mmol/L
9.	Hypertension with diastolic blood pressure ≥100 mmHg
10.	Heavy smokers (>10 cigarettes/day)
11.	Concurrent use of immunosuppressive drugs or fibrates
12.	Severe obesity with body mass index ≥30 kg/m^2
13.	A history of alcohol or drug abuse

obtained. The patient was allocated at random to either biweekly LDL-apheresis plus simvastatin (40 mg/day) or simvastatin (40 mg/day) alone. Randomization was stratified for total cholesterol and lipoprotein(a) (Lp(a)) levels, age, and coronary artery bypass graft status.

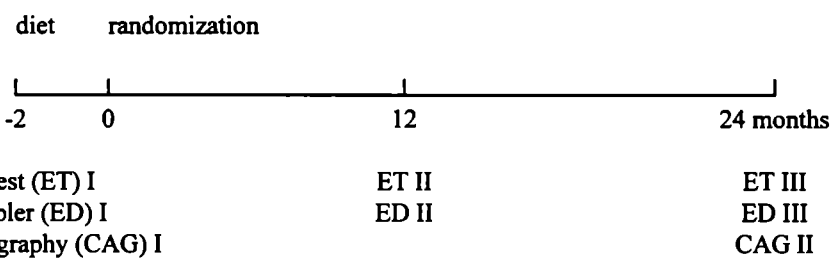
LDL-apheresis has been performed with an automated system with two small-sized dextran sulfate cellulose columns (MA-01 unit, Kanegafuchi Chemical industry Co., Japan). A plasma volume of 5000 mL was treated in each patient. The combination of LDL-apheresis and the HMG-CoA reductase inhibitor, simvastatin, can be expected to slow down the post-apheresis rebound in serum cholesterol due to increased cholesterol synthesis, which permits prolongation of the intervals between the apheresis procedures [55,56]. A resin in the highest tolerable doses will be added to the treatment if (pre-apheresis) serum total cholesterol levels in two consecutive months remains above 8.0 mmol/L.

Methods of follow-up

Lipids, lipoproteins and laboratory safety measures

Safety and tolerability of the treatment will be compared by means of medical and laboratory tests. In both groups, lipids, lipoproteins, blood chemistry, haematology, and routine urine analyses will be performed monthly. Apolipoprotein (apo)A1, apoB, and Lp(a) will be measured bimonthly. In the apheresis group, lipids and lipoproteins and some extra laboratory safety parameters will be measured biweekly, before and immediately after LDL-apheresis. At each visit the patients are subjected to a brief physical examination and dietary instruction is repeated frequently.

Serum total cholesterol and fasting triglycerides will be determined by enzymatic methods (CHOD-PAP, no. 237574, Boehringer Mannheim GmbH, FRG and Sera-PAK, no. 6639, Miles, Italy, respectively). HDL-C is determined using the polyethylene glycol 6000 precipitation method [57]. LDL-C is calculated by subtraction. Sam-



Randomization: LDL-apheresis *plus* medication or medication alone.

Fig. 1. Diagram of study design

ples for apoA1, apoB, and Lp(a) will be initially stored at -80°C, and determined at the end of the study. ApoA1 and apoB are quantified in serum by immunonephelometry and Lp(a) will be measured by a specific radioimmunoassay (apolipoprotein(a) RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden) [58].

Exercise tests

Replicate bicycle exercise tests (ET) will be performed at baseline, and after 12 months and 24 months. The ET is performed starting with a load of 50 W, and increasing the load every minute by 10 W during continuous ECG monitoring to maximum exercise. Blood pressure and 12-lead ECG registration will be monitored at rest, at maximum exercise, and every minute during the test. Automated calculation of ST-segment depression and slope is used, and data will be corrected for maximal systolic blood pressure heart rate product.

Coronary angiography

The coronary angiograms will be made at baseline and after 2 years of treatment with the same non-ionic iso-osmolar contrast agent, using the same cine-angiogram techniques (Siemens Bicor and Digitron-3 quantitative measurement software package). No pre-angiographic vasodilatory agents will be given other than the ones needed to ameliorate catheter-induced vasospasm. The same sequence of projections will be used preferably at the same time of day. A centimeter grid is filmed to adjust for pincushion distortion and the same kind and diameter of catheters will be used and measured with a micrometer after the procedure to maximize quality control. Twelve to 15 coronary segments will be filmed in 2 or 3 projections. Computer-assisted quantitative analysis (CMS) of the paired angiograms will be performed blinded and at the angiography reference laboratory of the University Hospital of Leiden [59,60]. Minimal obstruction diameter will be assessed as a measure for localized atherosclerosis, whereas mean segment diameter will be used as measure for diffuse changes. Additionally, blinded qualitative analysis (global score) of pairs of angiograms will be performed by a panel of three independent cardiologists. Differences in determination of percent stenosis will be settled by revised general agreement of the whole panel.

Videodensitometric assessment of coronary perfusion

Coronary arteriography will be followed by coronary flow estimation after maximal vasodilation with intracoronary administration of papaverine using digital subtraction techniques (Siemens Digitron-3). Contrast is injected using a power injector. Image acquisition will be performed while holding the breath at maximal inspiration and using the principle of apparent cardiac arrest by achieving synchronization of the X-ray pulses with the paced heart beats as has been described previously [40,41]. At least

5 regions of interest (ROI) will be selected in which measurement of coronary flow should be performed. These ROI's are located adjacent to the three main coronary arteries and an area selected distal to severe stenosis and after bypass grafts. Gamma-fitted time-density curves will be obtained by sampling the average pixel density within a ROI in the consecutive images and corrected by subtraction of the sampled average density in the corresponding background ROI. Data will be normalized for blood pressure values.

Measurement of the intima-media thickness (IMT) of the carotid artery
High resolution B-mode ultrasound examinations of the left and right carotid arteries are performed at baseline, and after 12 and 24 months of treatment, and are stored on videotape. An ACUSON 128XP duplex apparatus equipped with a 7.0-MHz L7384 linear array/ 5.0-MHz pulsed Doppler transducer combination is used. Measurement of the IMT is based on the distance between ultrasonically defined interfaces of the lumen/intima and media/adventitia. From a fixed latero-lateral angle the near and far wall of the common carotid artery, bulbus and internal carotid artery will be scanned. The

Table 2. Baseline characteristics (numbers or means±SD)

	LDL-apheresis (n=21)	Medication-only (n=21)
Age (y)	50.2±9.6	53.9±8.7
Weight (kg)	81.5±9.7	80.8±8.6
BMI ($kg.m^{-2}$)	26.6±2.0	26.2±2.0
Blood pressure		
systolic (mmHg)	129.3±17.3	126.3±18.1
diastolic (mmHg)	78.2±8.9	76.5±9.0
Smoking	3	4
Infarction	16	18
CABG	10	10
PTCA	2	5
Hypertension	2	5
Stroke	1	3
Claudication	3	5

Table 3. Baseline lipids and lipoproteins

	LDL-apheresis		Medication-only	
	mean±SD	range	mean±SD	range
Tot.Cholesterol (mmol/L)	9.72±1.84	7.9-14.3	9.85±2.21	7.8-16.6
Triglycerides (mmol/L)	2.50±1.23	0.83-5.93	2.65±1.44	0.66-5.29
HDL-Cholesterol (mmol/L)	0.95±0.19	0.60-1.45	0.93±0.21	0.64-1.33
LDL-Cholesterol (mmol/L)	7.72±1.96	5.73-12.62	7.85±2.36	5.44-14.55
Apolipoprotein A1 (g/L)	1.43±0.30	0.97-2.18	1.46±0.38	0.84-2.33
Apolipoprotein B (g/L)	2.59±0.48	1.70-3.50	2.60±0.63	1.40-4.20
Lipoprotein(a) (mg/dL)	57.0±63.4	2.9-254.1	38.4±39.4	1.7-138.6
median	30.0		20.4	

dilation of the common carotid artery and the flow divider in the carotid artery are used as anatomical landmarks. The blinded off-line quantification of the IMT of the arterial segments is assessed by a semi-automated contour detection program and is performed at the vascular laboratory of Interuniversity Cardiology Institute of the Netherlands (Utrecht).

Doppler spectrum analysis of the femoral artery and ankle/arm blood pressure ratio
These will be performed after a 1-year interval in the vascular laboratory of the University of Nijmegen. Doppler signals are obtained with an 8-MHz bidirectional continuous wave Doppler apparatus (Medasonics Inc., Mountain View, CA). Reactive hyperaemia is induced by thigh cuff compression for 5 minutes at a pressure of at least 50 mmHg above the systolic arterial thigh pressure and Doppler spectra will be obtained approximately 15s after relief of the thigh compression. Doppler signals are processed by a real-time spectrum analyser (Radionics SA8000; Scarborough, Ontario, Canada) and subsequently fed into a Digital MNC 11/23 computer on the basis of electrocardiographic triggering for off-line analysis. Maximum-frequency waveforms are calculated from the spectra by local convolution algorithm. To describe the shape of the waveforms, several parameters will be calculated [61]. Based on a combination of six of these Doppler parameters, the presence of haemodynamically significant aorto-iliac pathology can be assessed accurately [46]. The same Doppler probe is used to determine the ankle/arm pressure index. Haemodynamically significant vascular disease is defined as an ankle/arm pressure index at rest <0.90 and/or a decrease of the pressure

index during reactive hyperemia ≥0.20.

Baseline characteristics

In both treatment groups, 21 men were enrolled and some baseline characteristics are shown in Table 2. All patients had severe coronary atherosclerosis: a previous history of myocardial infarction was present in 16 vs 18 men in the apheresis and the medication group, respectively, and CABG had been performed in 10 men in both groups. In the apheresis group 17 of 21, and in the medication group 19 of 21 men, had 3-vessel CAD, whereas the other patients had 2-vessel disease. Haemodynamically significant lesions in the aorto-tibial tract were found in 4 and 5 men, and carotid artery segments with a mean IMT >1.0 mm were found in 29% and 22.8% in the apheresis and medication groups, respectively. Baseline cholesterol levels were very high, and predominant elevation of apoB-containing lipoproteins was found (Table 3).

Conclusion

LAARS will evaluate whether aggressive lipid lowering with LDL-apheresis in men with primary hypercholesterolaemia and extensive coronary artery disease will exert better retardation of the progression of atherosclerosis or even regression of coronary and peripheral atherosclerosis in comparison to conventional treatment. This study may help to answer the question whether extreme lipid lowering is indicated in this particular group of patients.

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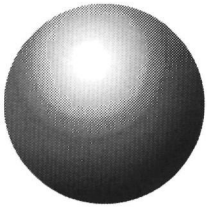

Chapter

Four

The rebound of lipoproteins after LDL-apheresis. Kinetics and estimation of mean lipoprotein levels

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The rebound of lipoproteins after LDL-apheresis. Kinetics and estimation of mean lipoprotein levels.

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We studied the rebound of lipoproteins in 20 hypercholesterolemic men [mean total cholesterol (TC) levels 9.6±1.8 mmol/L] after LDL-apheresis (LA) to determine the rate of recovery, the change in cholesterol synthesis, and to find a uniform estimation for time-averaged levels. After 10-20 months on biweekly LA using dextran sulfate cellulose columns and concomitant simvastatin administration time-averaged levels (±SD) measured by integration of the area under the curve were as follows: TC 4.4±1.0 mmol/L, LDL-cholesterol (LDL-C) 2.5±1.0 mmol/L, apolipoprotein B (apo B) 1.3±0.3 g/L, triglycerides (TG) 1.7±0.7 mmol/L, HDL-C 1.1±0.2 mmol/L, and lipoprotein(a) [Lp(a)] 53.7±49.4 mg/dL. Mean acute reductions in TC, LDL-C, apo B, Lp(a), and TG were 61%, 77%, 75%, 76%, and 62%, respectively. HDL-C levels were not influenced. Median recovery half times for TC, LDL-C, apo B, and Lp(a) were 3.0, 4.0, 2.3, and 3.5 days, respectively. The rebound of Lp(a) was identical to LDL-C, in 12 and 13 days post-treatment, respectively, whereas apo B and TC returned to pretreatment levels in 7.5 and 10 days, respectively, due to the fast rebound of VLDL particles. Notwithstanding these differences, time-averaged levels (C_{AVG}) could be estimated uniformly for the 4 latter parameters with the formula: $C_{AVG}=C_{MIN}+0.73(C_{MAX}-C_{MIN})$, where C_{MAX} and C_{MIN} are the immediate pre- and post-treatment levels. During long-term treatment the whole-body cholesterol synthesis was increased as measured by the ratio lathosterol to cholesterol of 3.60±1.45 μmol/mmol, whereas no further transient increase in the recovery period after LA was found. In conclusion, long-term LA and simvastatin therapy induced acute and chronic changes in lipids and lipoproteins showing the feasibility of biweekly treatment. It was shown that time-averaged levels, as a measure for the effective plasma levels, can be accurately estimated from pre- and post-treatment levels only.

Introduction

The performance of regular low density lipoprotein (LDL) apheresis permits the achievement of lower levels of LDL cholesterol which is usually not possible to attain with drug therapy alone [1,2]. Consequently, the application of LDL-apheresis may offer opportunities in the arrest of progression or regression of coronary and peripheral vascular disease in selected hypercholesterolemic patients [3-13]. Recently, the first two controlled LDL-apheresis regression studies were published, the FH Regression Study and the LDL-Apheresis Atherosclerosis Regression Study (LAARS) [12,13]. Both studies showed angiographical arrest of progression of coronary artery disease in severe hypercholesterolemic subjects concomitantly treated with an HMG-CoA reductase inhibitor, whereas the latter also showed reduction of the extent of peripheral vascular disease [14].

Treatment using selective LDL-apheresis removes large amounts of apolipoprotein B (apo B) containing lipoproteins from the body in a relatively short time, generally 2 - 4 h. Sawtooth-like alterations in lipoprotein levels are one of the most striking differences between patients undergoing repetitive LDL-apheresis and conventional therapy [15]. The efficacy of the treatment depends on the pre- and post-treatment lipid levels, and on the post-treatment return of lipids in plasma [16-20]. The combination therapy of LDL-apheresis and an HMG-CoA reductase inhibitor can be expected to decrease pretreatment LDL cholesterol to lower levels and slow down the post-apheresis rebound, which permits prolongation of the intervals between the apheresis procedures [17,20]. It has been shown that the increase of lipoprotein levels after the treatment can be explained by first order kinetics [16,21]. Therefore, it is accepted that time-averaged concentrations provide the best estimate of the physiologically effective plasma levels during long-term treatment with LDL-apheresis.

There is no universal agreement whether the cholesterol synthesis is increased after a single apheresis or during regular treatments. An increased rate of cholesterol biosynthesis without any change in apo B synthesis has been reported after plasmapheresis [22-24], whereas both a transient increase and no change in cholesterol synthesis have been shown after LDL-apheresis [19,20,25,26].

In this paper, we measured the rebound of lipids and lipoproteins in days following a treatment during long-term LDL-apheresis using dextran sulfate cellulose columns (DSC) [27,28]. Changes in the amounts of lipids and lipoproteins were measured, and the absolute and fractional recovery rates were calculated. Changes in cholesterol biosynthesis and in intestinal adsorption rate were quantitated indirectly by measurements of the sterol intermediate lathosterol and the plant sterol sitosterol in relation to serum cholesterol, respectively. The data were used for estimation of the mean effective lipid or lipoprotein concentrations with a formula during long-term biweekly treatment in LAARS [13].

Materials and methods

Subjects and treatment

The present study was carried out in subjects randomized to treatment with LDL-apheresis in the LDL-Apheresis Atherosclerosis Regression Study (LAARS) [13]. In this study the effect of 2 years of cholesterol lowering using biweekly LDL-apheresis plus simvastatin treatment was compared with the effect of conventional lipid lowering with simvastin alone on coronary and peripheral vascular disease. All eligible LAARS participants were men with a primary hypercholesterolemia and extensive, angiographically assessed severe coronary artery disease. This study was approved by the ethical committee of the University Hospital of Nijmegen. Twenty-one subjects were enrolled for long-term LDL-apheresis of whom 20 men gave informed consent for the present study. All subjects, age 50 ± 9 years, range 30-66, were classified as heterozygous for familial hypercholesterolemia. Baseline lipids and lipoproteins before the start of the trial on a standard lipid lowering diet were as follows: total cholesterol 9.64±1.78 mmol/L, triglycerides 2.40±1.04 mmol/L, LDL cholesterol 7.66±1.92 mmol/L, high density lipoprotein (HDL) cholesterol 0.96±0.18 mmol/L, lipoprotein(a) [Lp(a)] 59.7±64.3 mg/dL [median 34.7, range 3.1 - 236.3], and apo B 2.55±0.43 g/L.

Concomitant treatment with the HMG-CoA reductase inhibitor simvastatin (40 mg/day) was started at the beginning of the study. LDL-apheresis was performed fortnightly with an automated system equipped with 2 small-sized dextran sulfate cellulose columns (Liposorber®) in combination with a membrane type plasma separator (MA-01 unit, Kanegafuchi Chemical Industry Co. Ltd., Osaka, Japan). During this extracorporeal procedure blood was anticoagulated with heparin at a rate of 2,000 U/h. A volume of 5000 mL (approximately 1.5 plasma volumes) was treated per session, which lasted 3 to 4 h at a blood pump rate ranging between 80 and 120 mL/min. The present experiments were performed for every subject on one occasion, 10 to 20 months after the start of the first apheresis.

Rebound curves

In order to follow the return of the various lipids and (apo)lipoproteins to their pretreatment values blood samples were taken immediately before and immediately after apheresis, and at 24 h intervals subsequent to the apheresis for a period of seven days, and on the 10th and 14th day post-apheresis. For construction of the rebound curve additional data of immediate pre- and post-treatment levels from 4 forgoing apheresis procedures were used to reduce the individual variability. The coefficient of variation of these pre- and post-treatment levels was within total analytical and biological variance.

Analytical procedures

All blood samples, except those taken immediately after apheresis, were obtained between 8:00 and 9:00 h by venapuncture after an overnight fast. The post-apheresis samples were drawn between 13:00 and 14:00 h. For the indirect assessment of whole body cholesterol biosynthesis in vivo we used the quantification in serum of the cholesterol intermediate lathosterol, and more specifically the lathosterol to cholesterol ratio [29-31]. Serum concentrations of lathosterol are directly associated with hepatic HMG-CoA reductase activity [32]. Serum lathosterol concentration and the lathosterol to cholesterol ratio are independent of the composition of diet [30], but are increased during treatment with resins [33] and decreased during therapy with HMG-CoA reductase inhibitors [30,34] reflecting corresponding changes in cholesterol biosynthesis in the liver. Plasma concentrations of the plant sterol β-sitosterol were also measured. Sitosterol levels and especially the sitosterol to cholesterol ratio reflect the balance between sterol absorption and biliary excretion rate, and are closely related to the fractional absorption of dietary cholesterol [35,36]. Plasma lathosterol and sitosterol levels were measured by high-performance liquid chromatography (HPLC Spectra Physics model 8800, Breda, The Netherlands), essentially as reported before, and shown in μmol/L and also as μmol/mmol cholesterol to correct for the effects of apheresis in removing lipoproteins [30]. Plasma cholesterol and triglycerides were determined with commercially available enzymatic methods (Boehringer Mannheim, FRG, Nos. 237574, and Sera-PAK, Miles, Italy, no. 6639, respectively). To determine plasma HDL cholesterol the polyethylene glycol 6000 precipitation method was used [37]. LDL cholesterol was calculated by the Friedewald formula. Samples for apo B and Lp(a) were initially stored at -80°C, and determined at the end of the study. Apo B was quantified by immunonephelometry [38] and recalculated on the basis of data from exchange of sera with the North West Lipid Research Clinic (Seattle, USA). Lp(a) was be measured by a specific radioimmuno-assay [apolipoprotein(a) RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden].

Statistical procedures

Assuming a one-compartment model and semi-steady state conditions, the rate of return to pretreatment cholesterol concentration can be predicted from the following equation: $C_T=C_{MAX}-(C_{MAX}-C_{MIN})e^{-kT}$, where T is the time in days after the treatment, and C_{MAX} and C_{MIN} represent the levels immediately pre- and post-apheresis [16]. The first-order turnover constant k or the fractional catabolic rate (FCR) can be estimated by curve-fitting using nonlinear regression (first order reappearance curve), i.e. a least squares regression analysis. The time in days to regain 50% and 90% of the eliminated amount was derived from k, since T50%=0.69/k and T90%=2.30/k. When in this first-order model the lipoprotein pool is acutely depleted, the subsequent asymptotic recovery is characterized by a constant absolute inflow and a constant fractional outflow of lipo-

Table 1. Change in plasma lipids and lipoproteins

	TC (mmol/l)	TG (mmol/l)	LDL-C (mmol/l)	HDL-C (mmol/l)	Lp(a) (mg/dl)	apo B (g/L)
C_{MAX}	5.36±1.22	1.76±0.77	3.48±1.20	1.07±0.21	60.6±67.5	1.53±0.37
C_{MIN}	2.07±0.50	0.46±0.20	0.79±0.44	1.06±0.19	14.2±14.6	0.39±0.13
% change	-61±5*	-62±9%*	-77±6%*	-2±7	-76±13%*	-75±6%*
C_{AVG} (AUC)	4.36±0.95	1.69 ±0.73	2.47±0.97	1.07±0.20	53.7±49.4	1.28±0.27
C_{AVG} (formula)	4.44±1.16	-	2.57±1.08	-	57.9±57.2	1.24±0.35

*Data represent means±SD (n=20). TC, total cholesterol; TG, triglycerides; L(H)DL-C, low (high) density lipoprotein cholesterol; Lp(a), lipoprotein(a); apo B, apolipoprotein B; C_{MAX} and C_{MIN}, levels immediately before and after 5 consecutive aphereses; C_{AVG}, time-averaged levels, as measured by integration of the area under the rebound curve (AUC) or estimated by a formula (see text); %change versus C_{MAX} : *P<0.001 (t-test or Mann-Whitney U-test were appropriate)*

proteins [16,22,23,26]. The absolute recovery rate was calculated by multiplying the change in pool size with k, and expressed relatively to body weight (mg/kg/day). For estimation of the size of the removed pools, plasma volumes were calculated with the formula: V_{PLASMA}= (1-hematocrit [l/l]).weight[kg]/13.

Time-averaged concentrations (C_{AVG}) were measured in each patient by integration of the area under the rebound curve. Since C_{AVG} is determined under first-order conditions by C_{MAX} and C_{MIN} and a constant α in the formula $C_{AVG}= \alpha C_{MAX}+(1-\alpha)C_{MIN}$, we were able to calculate α for all procedures from α=(Cavg-Cmin)/(Cmax-Cmin) [39]. To investigate whether time-averaged levels of total cholesterol, LDL cholesterol, Lp(a), and apo B could be estimated correctly by using this simple formula, we used a median α for all four parameters. Time-averaged levels calculated with this formula were then compared with those measured by integration under the curve.

Analyses were performed with procedures available in the software package of SPSS (SPSS Inc., Chicago, IL.). Multivariate analysis of variance (ANOVA), followed by *t*-tests were used for normally distributed data, whereas Wilcoxon's signed ranks tests were used for differences in means of not normally distributed data. For measurements of agreement the Pearson product-moment correlation coefficient was used. A two-sided p-value of less than 0.05 was considered to be significant. Results are expressed as means ± SD, unless otherwise indicated.

Results

Acute reductions in plasma lipids and lipoproteins

During biweekly treatment with LDL-apheresis and simvastatin lipid and lipoprotein concentrations were reduced to the pretreatment levels (C_{MAX}), as shown in Table 1. LDL-apheresis acutely reduced plasma total cholesterol and triglycerides to the same extent, 61% and 62%, respectively, whereas LDL cholesterol, apo B, and Lp(a) were lowered further and also to the same extent by an average of 77%, 75%, and 76%, respectively. HDL cholesterol levels were not influenced by LDL-apheresis. Immediate post-treatment levels (C_{MIN}) of all apo B-containing lipoproteins were below 10% limit values of a normal population, as shown in Table 1. The mean removed amounts in grams by a single apheresis are shown in Table 2. A good correlation was found between the removed amounts of lipids and lipoproteins and the initial amount in total plasma volume, with *r* ranging from 0.96 to 0.99 ($P<0.001$) for total cholesterol, triglycerides, LDL cholesterol, apo B, and Lp(a). This is shown exemplarily for LDL cholesterol in Figure 1, based on 5 consecutive procedures in all 20 patients. Generally, this indicates that the higher the plasma concentration of LDL cholesterol, the higher is the removed mass, given a constant treated plasma volume of 5000 mL.

Table 2. Kinetics of recovery after a single LDL-apheresis during treatment with simvastatin

	TC	LDL-C	Lp(a)	apo B
removed pool (g)	4.51±1.72 (4.03)	3.54±1.62 (3.03)	2.04±2.18 (1.53)	4.20±1.35 (3.95)
k (pools/day)	0.24±0.08 (0.23)	0.19±0.08 (0.18)	0.24±0.18 (0.20)	0.32±0.17 (0.31)
$T_{50\%}$ (days)	3.2±1.0 (3.0)	4.3±2.2 (4.0)	3.9±1.9 (3.5)	2.5±0.9 (2.3)
$T_{90\%}$ (days)	10.5±3.5 (10.0)	14.4±7.4 (13.2)	12.9±6.3 (11.8)	8.4±3.1 (7.5)
Abs. recovery rate (mg/kg/day)	12.9±4.5 (12.7)	8.2±4.7 (7.4)	4.7±4.9 (3.5)	16.4±9.9 (14.0)

Data represent mean±SD and median levels between brackets (n=20). TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; Lp(a), lipoprotein(a); apo B, apolipoprotein B; k, first-order turnover constant; $T_{50\%}$ and $T_{90\%}$, time for resynthesis of 50% and 90% of the eliminated concentration; abs. recovery rate, absolute recovery rate

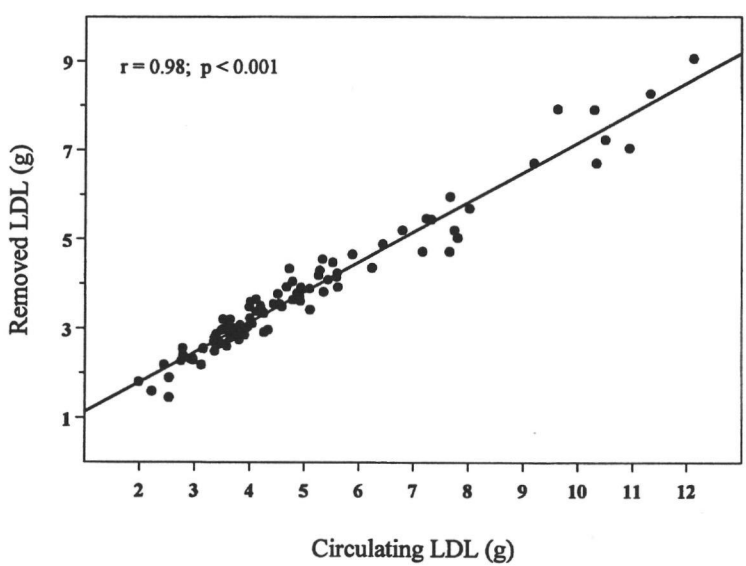

Fig. 1. Low density lipoprotein (LDL) cholesterol removed (g) by LDL-apheresis using dextran-sulfate cellulose columns in relation to the circulating amounts (g) in 5 consecutive procedures of 20 patients

Description of the rebound curves

The recovery after LDL-apheresis of the various lipoproteins was different. Triglycerides recovered rapidly, reaching 83% of the pretreatment levels within 1 day after treatment. Therefore, time-averaged levels of triglycerides were not significantly different when compared to pretreatment concentrations, 1.69±0.73 versus 1.76±0.77 mmol/L, respectively (P=0.21). The recovery of the other lipid and lipoproteins was much slower. The time for recovery of 50% of the removed amount (T50%) was between 2-4 days, followed by a successive slower rise indicated by the time for recovery of 90% of the eliminated amount (T90%) (Table 2). The median values for the first-order turnover constant k of total cholesterol, LDL cholesterol, Lp(a), and apo B were 0.23, 0.18, 0.20, and 0.31, respectively, showing the fastest recovery of apo B and an intermediate position for total cholesterol. The rebound curves of Lp(a) and LDL cholesterol, when expressed as a proportion of the removed amount of lipoprotein, were not significantly different (Fig. 2). The absolute recovery rate (Table 2) was lowest for Lp(a), subsequently followed by LDL cholesterol (compared with Lp(a): P=0.03), total cholesterol (compared with LDL: P=0.006), and apo B (compared with total cholesterol: P=0.08). Pearson's correlation coefficients between the removed amount and the absolute recovery rate were as follows: total cholesterol r=0.45 (P=0.04), LDL cholesterol r=0.64 (P=0.002), Lp(a) r=0.74 (P=0.001), and apo B r=0.17 (P=0.48). This generally indicated a higher absolute recovery rate of LDL cholesterol and Lp(a) in patients with high pretreatment levels.

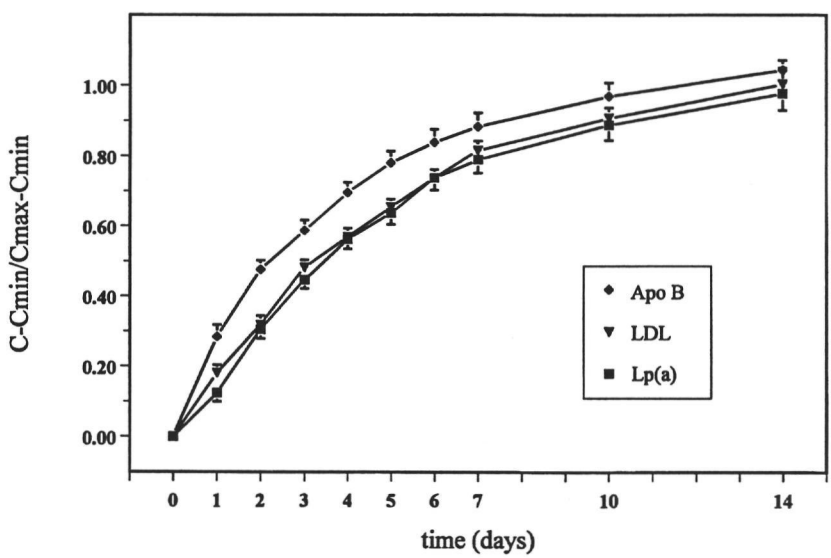

Fig. 2. Recovery of apolipoprotein B (apo B, diamonds), low density lipoprotein cholesterol (LDL, triangles) and lipoprotein(a) (Lp(a), squares) in days after LDL-apheresis expressed as ratio of the removed amount of lipoproteins (n=20)

Table 3. Mean plasma total lathosterol and sitosterol levels immediately before and after LDL apheresis during treatment with simvastatin

	Lathosterol (μmol/L)	Latho/Chol (μmol/mmol)	Sitosterol (μmol/L)	Sito/Chol (μmol/mmol)
Before	18.03±7.13	3.60±1.45	15.73±7.66	3.03±1.08
After	6.40±3.42	3.41±1.90	6.60±3.01	3.34±1.13
%change	-67±14*	-10±23	-57±9*	+14±26

*Data represent means±SD of 2 procedures in 12 patients. Latho/Chol, lathosterol to cholesterol ratio; Sito/Chol, sitosterol to cholesterol ratio. Normal values in a sex-, age-, and cholesterol-matched population: lathosterol 7.43±2.84 μmol/L, sitosterol 6.78±2.88 μmol/L (n=158) [31]. Differences: *P<0.001 (t-test)*

Time-averaged concentrations

Time-averaged (C_{AVG}) lipid and lipoprotein levels, calculated by integrating beneath the rebound curve, are shown in Table 1. These concentrations could be uniformly estimated by the formula mentioned in the method section. Mean levels of α for total cholesterol, LDL cholesterol, apo B, and Lp(a) were 0.72±0.09, 0.70±0.09, 0.78±0.10, and 0.68±0.11, respectively. The application of the overall median α=0.73 showed very good correlations between both methods of calculation of C_{AVG} for all four parameters with correlation coefficients r ranging from 0.96 to 0.99 (P<0.001).

Measurements of plasma lathosterol and sitosterol levels

Pre- and post-treatment lathosterol and sitosterol concentrations, only measured in 12 patients, are shown in Table 3. Pretreatment levels and ratios to cholesterol were high, when compared to normal ranges in men with the same age and cholesterol levels [31], indicating an overall increased whole-body cholesterol synthesis in these patients on long-term apheresis and a cholesterol synthesis inhibitor. After a single apheresis a significant reduction of 67% in lathosterol and 57% in sitosterol levels were found. However, when expressed relatively to cholesterol no significant changes in plasma total lathosterol and sitosterol levels were observed immediately after apheresis. Moreover, multivariate ANOVA of the recovery curves in days after apheresis (data not shown) of both lathosterol to cholesterol ratio and sitosterol to cholesterol ratio showed no statistically significant changes compared to pretreatment levels, P=0.44 and P=0.37, respectively, indicating no further increase in cholesterol synthesis nor in cholesterol absorption rate in the days post-apheresis.

Discussion

The aim of the present study was to examine acute changes induced by a single apheresis procedure during chronic treatment. It was demonstrated that LDL-apheresis induced substantial reductions in plasma lipids and lipoproteins, more than has been shown by others using DSC columns, immunoadsorption, or HELP (Heparin Extracorporeal LDL Precipitation) [8-12]. Dependent on the initial levels, apheresis selectively removed large amounts of apo B-containing lipoproteins from the body, including VLDL, roughly 5 to 6% of the total exchangeable cholesterol body stores [40]. The post-treatment rebound of serum triglycerides was very fast, reaching pretreatment values within 1 to 2 days, whereas the recoveries of apo B, total cholesterol, LDL cholesterol, and Lp(a) during concomitant therapy with simvastatin ranged between 8 and 13 days, and were not associated with a post-treatment increase in whole-body cholesterol synthesis or intestinal cholesterol absorption.

Long-term turnover studies using radio-labelled cholesterol have suggested the existence of three body pools of cholesterol [40]. After depletion of the rapid exchangeable pool, represented by e.g. plasma lipoproteins, erythrocytes, liver, and intestines, recuperation of this pool is mediated by input via an intermediate exchangeable pool (e.g. skin and adipose tissues) and a slowly exchangeable pool (e.g. skeletal muscles and arterial wall). In addition, cholesterol homeostasis will also be corrected by increased cholesterol synthesis and intestinal absorption. Using stable isotope methodology Arends *et al* [26] have shown no increase in the absolute hepatic VLDL apo B production as well as LDL apo B production in normal volunteers after a single LDL-apheresis. Among others [24], these latter authors plausibly showed a constant absolute apo B synthesis versus a constant fractional catabolic rate independent of the change in pool size post-apheresis, which concords with a simple one-compartment model as assumed by Apstein *et al* [16]. Analyses of such models reduces complexity, but have also been shown to yield similar results as multi-compartment models [41]. Moreover, values for k calculated as described in the method section using a simple nonlinear model of asymptotic regression, have been shown to be comparable to those measured in steady state conditions during turnover studies with radio-labelled isotopes [16,42].

Comparison of the absolute recovery rates and the recovery half-times (T50%) clearly showed the highest rebound for apo B and total cholesterol. This must be explained by the very fast recovery of triglycerides, in particular VLDL particles. It has been reported that the absolute production rate of VLDL apo B is twice that of LDL apo B and returns to baseline 16 h after apheresis [26]. The major apolipoprotein of LDL, apo B-100, is predominantly synthesized in the liver and enters the circulation as a component of VLDL [43,44]. Conversion of VLDL to LDL and thus transfer of apo B to LDL takes place through lipolysis. Since we did not observe a post-treatment increase in intestinal absorption, i.e. sitosterol to cholesterol ratio, the fast increase in

triglycerides after apheresis reflects de novo VLDL synthesis. This explains that T50%-values for apo B and total cholesterol are lower than for LDL, because they also reflect the rapid increase in VLDL particles, which are not present in the LDL fraction.

We did not observe a different rebound between LDL cholesterol and Lp(a), as has been shown by others [12,47]. Median values for constant k of LDL cholesterol in our study were lower than what has been shown by others [45-47] ranging between 0.22 and 0.23, which is probably associated with the more effective inhibition of HMG-CoA reductase using simvastatin [17,47]. As indicated by median k-value of 0.14 for Lp(a), Armstrong *et al* [45] observed a slower rebound of Lp(a) in comparison to LDL, whereas Koizumi *et al* [47] found a faster recovery for Lp(a). However, the recovery curves of the latter author were only based on 3 time points. Taking into account the skewness of the data, the more appropriate median figure for k of Lp(a) in this latter study of 0.19 indicates rather a slower rebound when compared to the median for LDL of 0.22. Therefore, that there is no reason to believe that the rebound of Lp(a) is faster than that of LDL cholesterol.

Body stores would be quickly depleted if not replaced by absorption of dietary cholesterol or newly synthesized cholesterol. Parker [19] and Pfohl *et al* [20] have shown evidence for transiently increased endogenous cholesterol synthesis after LDL-apheresis. In agreement with Gylling *et al* [25], however, we did not find an increase in cholesterol synthesis nor in intestinal absorption. These different observations may be explained by the fact that we measured the rebound during ongoing long-term biweekly treatments, whereas several other investigators have analyzed the recovery of lipids and lipoproteins after a single procedure [19,26,45,47]. Indeed, we observed high pretreatment ratios for plasma lathosterol and sitosterol relative to cholesterol, indicating an upregulated cholesterol synthesis and intestinal absorption before the start of the analysis of the rebound. Since it has been shown that long-term therapy with HMG-CoA reductase inhibitors induced a reduction or no change in the rate of whole-body cholesterol synthesis [48,49] the lack of a further transient post-treatment increase in cholesterol synthesis post-apheresis is reasonably explained by the yet increased rate of synthesis, due to the many forgoing procedures. However, Pfohl *et al* [20] also studied their patients under long-term LDL-apheresis. Their treatment was not as effective as ours, since pretreatment total cholesterol and LDL cholesterol after 12 months of apheresis were still 7.4±0.3 and 5.5±0.3 mmol/L, respectively. Besides, it was accompanied by a gradual, although not significant, increase in lathosterol to cholesterol ratio and mevalonic acid, and changes in levels of these cholesterol precursors were negatively correlated with the post-apheresis LDL cholesterol level. So, more aggressive lipid lowering in our study may have induced a yet increased pretreatment cholesterol synthesis, preventing further transient post-treatment increments.

The continuous rise and fall of plasma lipoproteins necessitates the estimation of the time-averaged levels, since the mean of pre- and post-treatment concentrations

overestimates effective plasma levels [12]. We showed that the estimation of time-averaged levels of total cholesterol, LDL cholesterol, apo B, and Lp(a) could be accurately performed by a simple uniform formula using only pre- and post-treatment levels, $C_{AVG}=C_{MIN}+0.73(C_{MAX}-C_{MIN})$, notwithstanding the differences in post-treatment rebound. Theoretically, this formula is not specific for the conditions we used during the study, i.e. biweekly procedures and a treated plasma volume of 5000 mL. This should, however, be evaluated first before this formula is used under different conditions.

In conclusion, our study showed that chronic, aggressive lipid lowering with LDL-apheresis stimulated cholesterol biosynthesis, which was not prevented by concomitant treatment with simvastatin. Biweekly procedures were not followed by a transient further increase in cholesterol absorption or whole-body synthesis. The fast rebound of the VLDL pool determined a quicker recovery of total cholesterol and apo B, whereas the return to pretreatment levels of Lp(a) and LDL cholesterol in 12 and 13 days, respectively, showed that biweekly apheresis procedures, treating 5000 mL of plasma, is an acceptable strategy.

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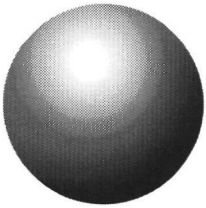

Chapter

F i v e

The rebound of lipoproteins after LDL-apheresis. Effects on chemical composition and LDL-oxidizability

Submitted for publication as:

The rebound of lipoproteins after LDL-apheresis. Effects on chemical composition and LDL-oxidizability.

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The changes in low density lipoprotein (LDL) composition and oxidizability after LDL-apheresis (LA) using dextran sulfate cellulose columns were evaluated in 12 hypercholesterolemic men [mean (±SD) total cholesterol (TC) 9.7±1.8 mmol/L]. After 10-20 months on biweekly LA and 40 mg per day simvastatin immediate pre-apheresis levels of TC, LDL-cholesterol, and apolipoprotein B were decreased to 5.3±1.3 mmol/L, 3.3±1.2 mmol/L, and 1.6±0.4 g/L, respectively, whereas apheresis induced mean acute reductions of 61%, 78%, and 76%, respectively. Measurements of copper-induced LDL-oxidizability in vitro showed an increased resistance against oxidation after LA until day 3 post-treatment: lag time (min) [day 0 (before LA) vs day 1 (post-LA)] 112±27 vs 130±26 (P=0.001), maximal rate of diene production (nmol/min/mg LDL) 11.1±2.7 vs 9.1±2.1 (P=0.001), and time to maximal diene production (min) 186±39 vs 209±35 (P=0.001). Analysis of the chemical composition of LDL revealed a 25% (P<0.001) reduced content of cholesteryl esters and a decrease of the cholesterol to protein ratio of 1.20±0.25 to 0.70±0.22 (P<0.001) through the 3rd day post-LA. Linoleic acid and arachidonic acid content of LDL decreased 11 and 18%, respectively, at the expense of palmitic acid. Vitamin E levels (mg/L) were significantly lowered due to reduction of the lipoprotein pool by apheresis, however, vitamin E content of LDL did not change in the days after apheresis when expressed per g protein or per µmol linoleic acid. The changes in fatty acid pattern were strongly associated with changes in LDL-oxidizability indices ($P \leq 0.01$). Thus, LA effectively decreased LDL pool size, inducing the presence of less buoyant lipoproteins, which were less susceptible to in vitro oxidation. This was not explained by changes in vitamin E levels, but by short-term changes in the fatty acids composition.

Introduction

Progression of coronary and peripheral atherosclerosis can be retarded or even regressed by effective cholesterol lowering [1,2]. Continuous LDL-apheresis with dextran sulfate cellulose as adsorbent selectively removes apolipoprotein (apo) B containing lipoproteins from the plasma [3]. This procedure has gained interest in the treatment of hypercholesterolemic patients, especially those refractory to drug treatment and with extensive coronary artery disease [4-6]. The efficacy of this treatment depends on the pre- and post-treatment lipid levels, and on the post-treatment return of lipids in plasma [7-10].

Evidence exists to suggest that oxidative modification of low density lipoproteins (LDL) plays a key event in the development of atherosclerosis. It has been shown that LDL must undergo oxidative modification before it can be taken up by macrophages [11-13]. Oxidative modification of LDL converts it to a form recognized by the macrophage scavenger receptor [14], leading to its unlimited uptake by cells. The presence of oxidized LDL and lipid peroxides in areas of atherosclerotic plaques and the observations that antioxidant treatment with probucol and vitamin E slows the progression of atherosclerosis in the animal model strongly support the role of LDL modification in atherogenesis [15-19]. However, in the animal model a decrease in oxidative susceptibility alone is not sufficient to attenuate atherogenesis when cholesterol levels remain markedly elevated [20,21]. Apart from the antioxidant content of LDL, susceptibility of LDL to oxidation is determined by the chemical composition and fatty acid content of LDL [22-27]. Changes in the relative cholesterol and protein content may lead to conformational changes in the core of the particle, possibly resulting in a different exposure of fatty acids to oxygen radicals and a decreased rate of lipid peroxidation [28].

Data on compositional changes of lipoprotein particles after apheresis are scarce [29-31]. It has been reported that particles of the high density lipoprotein $(HDL)_3$ subfraction become enlarged and enriched with free cholesterol and phospholipids, whereas the composition of the HDL_2 is only slightly modified [30]. Jadhav and Thompson reported the normalization of the cholesterol to phospholipid ratio in familial hypercholesterolemic patients within 1 day after plasma exchange, reversing to the pretreatment state in 2 to 3 weeks [29]. The lipid composition of the LDL fraction after apheresis has been reported only once [31]; the authors of this study showed that immediately post-apheresis LDL particles had more surface than core constituents and were also structurally different in comparison to pretreatment.

Since LDL-apheresis induces major changes in the cholesterol pool, which may affect the conformation of LDL and consequently influence LDL-oxidizability, we analyzed in the present study the acute changes in in-vitro oxidizability of LDL in days after LDL-apheresis in relation to its compositional changes. This opportunity was offered by a controlled study in our center which investigated the effect of more aggres-

sive cholesterol lowering on atherosclerosis using long-term treatment with LDL-apheresis [6].

Materials and methods

Subjects and treatment

The present study was carried out in subjects randomized to treatment with LDL-apheresis in the LDL-Apheresis Atherosclerosis Regression Study (LAARS) [6]. In this study the effect of 2 years of more aggressive cholesterol lowering using biweekly LDL-apheresis plus simvastatin treatment was compared with the effect of conventional lipid lowering with simvastatin alone on coronary and peripheral vascular disease. All eligible LAARS participants were men with a primary hypercholesterolemia and extensive, angiographically assessed coronary artery disease. Twenty-one subjects were enrolled for long-term LDL-apheresis of whom 12 (age 50±10, range 31-64 years) gave informed consent for the present study. Baseline lipids and lipoproteins (C_{BAS}) on a standard lipid lowering diet are given in Table 1, showing a predominant elevation of LDL cholesterol and apo B according to the inclusion criteria of the study. Lipoprotein(a) [Lp(a)] levels showed a skewed distribution (median 30.0, range 2.9 to 254.1 mg/dL). During the study all subjects continued their lipid lowering diet and were treated with the HMG-CoA reductase inhibitor simvastatin (40 mg/day). LDL-apheresis was performed fortnightly with an automated system equipped with 2 small-sized dextran sulfate cellulose columns (Liposorber®) in combination with a membrane type plasma separator (MA-01 unit, Kanegafuchi Chemical Industry Co. Ltd., Osaka, Japan). For this extracorporeal procedure blood was anticoagulated with heparin at a continuous flow of 2,000 U/h. A volume of 5000 mL (approximately 1.5 plasma volumes) was treated per session. All subject were also on concomitant treatment with anti-anginal drugs and acetylsalicylic acid in most cases.

During the study a rebound curve was constructed for every subject on one occasion in a semi-steady state phase, 10 to 20 months after the start of the study. Lipids and lipoproteins, oxidizability, chemical composition, fatty acid composition, and vitamin E content of LDL were determined immediately before and after apheresis, and on days 1, 3, 7, and 10 after apheresis.

Plasma samples

Blood samples were obtained by venapuncture after an overnight fast and collected into evacuated EDTA-containing (1 g/L) tubes. After centrifugation at room temperature (10 minutes at 2200*g*), 5 mL plasma samples were collected in polypropylene vials to which 50 µL 600 g/L saccharose was added as cryopreservant to prevent LDL aggregation. The vials were stored at -80°C until use.

Lipoprotein isolation

Very low density lipoproteins (VLDLs) plus intermediate density lipoproteins (IDLs) (d<1.019 g/mL) and LDLs (1.019<d<1.063 g/mL) were isolated by sequential ultracentrifugation, essentially as described by Havel et al [32]. LDL was dialyzed in the dark for 24 hours at 4°C against 3 L phosphate-buffered saline containing 10 μmol/L EDTA. The buffer was made oxygen-free by vacuum degassing followed by purging with nitrogen. The LDL containing solution was filtered through a 0.45 μm filter.

LDL oxidation

The in vitro oxidation of LDL was performed as described previously [25]. In short, 10 μmol/L EDTA-containing dialyzed LDL (adjusted to 0.5 mg LDL protein/mL) was diluted directly before the start of the oxidation with EDTA-free phosphate buffer to a final concentration of 0.05 mg/mL. This reduces the final EDTA concentration in the cuvette to 1 μmol/L. Oxidation was initiated by addition of a freshly prepared $CuCl_2.2H_2O$ solution (final concentration 15 μmol/L). The kinetics of LDL oxidation were determined by measuring the formation of conjugated dienes by monitoring the change in the 234 nm absorbance at 30°C on a Perkin-Elmer Lambda 5 UV spectrophotometer equipped wit a six-position automatic sample changer, allowing for the determination of up to 6 samples at the same time. Absorbance was recorded every 3 minutes for at least 5 hours. All measurements were performed in duplicate.

The kinetics of the absorbance profile of each individual LDL preparation was described by the following indices: the initial 234 nm absorbance ($diene_O$, expressed as nmol/mg LDL protein); the lag time, defined as the interval between the intercept of the linear least-square slope of the curve with the 'initial absorbance axis', expressed in minutes; the maximal rate of oxidation (rate), calculated from the slope of the absorbance curve during the propagation phase, expressed as nmol/min/mg LDL protein; the maximal amount of dienes formed ($diene_{MAX}$); and t_{MAX}, the time needed to reach maximal absorbance, expressed in minutes. The amount of dienes produced was calculated by subtraction of $diene_O$ from $diene_{MAX}$.

Other methods

Plasma total cholesterol, free (unesterified) cholesterol, phospholipids, and triglycerides in LDL samples were determined with commercially available enzymatic methods (Boehringer Mannheim, FRG, Nos. 237574, 310328, and 691844; and Sera-PAK, Miles, Italy, no. 6639, respectively). LDL protein was measured by the method of Lowry et al [33]. Plasma HDL cholesterol was determined using the polyethylene glycol 6000 precipitation method [34]. LDL cholesterol was calculated by subtraction. Apo B was quantified by immunonephelometry [35] and recalculated on the basis of data from exchange of sera with the North West Lipid Research Clinic (Seattle, USA).

Table 1. Change in plasma lipids and lipoproteins

	TC (mmol/l)	TG (mmol/l)	LDL-C (mmol/l)	HDL-C (mmol/l)	Lp(a) (mg/dl)	apo B (g/L)
C_{BAS}	9.72±1.84	2.32±1.03	7.78±1.86	0.93±0.18	57.0±63.9	2.59±0.47
C_{MAX}	5.28±1.30	1.75±0.88	3.25±1.22	1.08±0.23	60.8±68.0	1.58±0.40
C_{MIN}	2.06±0.51	0.51±0.28	0.73±0.43	1.06±0.22	13.8±14.4	0.39±0.15
change	-61±6%*	-68±15%*	-78±6%*	-2±7%	-72±12%*	-76±12%*
C_{AVG}	4.17±0.91	1.67±0.71	2.28±0.72	1.08±0.20	45.4±52.3	1.20±0.23

*Data represent means±SD of 12 patients. TC, total cholesterol; TG, triglycerides; L(H)DL-C, low (high) density lipoprotein cholesterol; Lp(a), lipoprotein(a); apo B, apolipoprotein B; C_{BAS}, baseline concentrations; C_{MAX} and C_{MIN}, levels immediately before and after apheresis; time-averaged concentrations (C_{AVG}) were calculated by integral calculation of the area under the rebound-curve. Changes versus C_{MAX} (absolute and proportional): *P<0.001 (t-test or Mann-Whitney U-test were appropriate)*

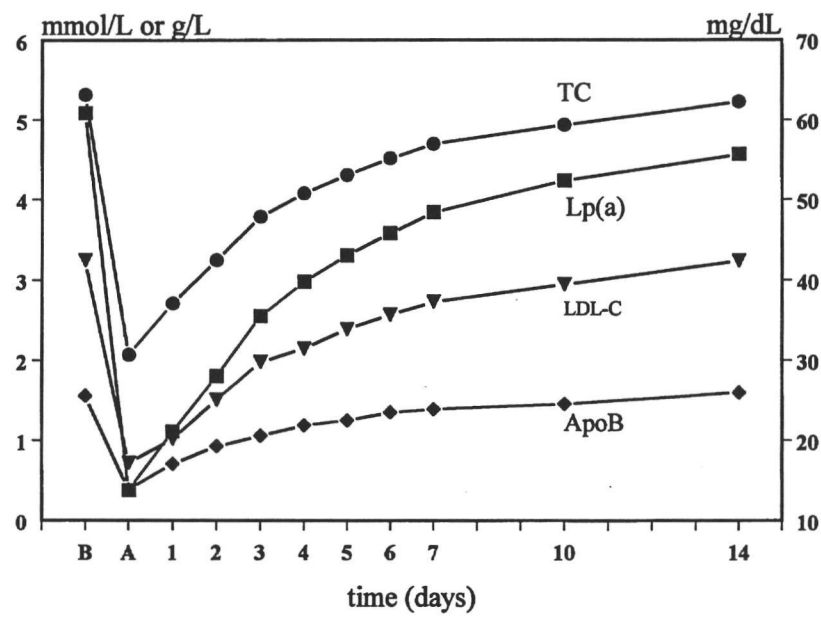

Fig. 1. Recovery of plasma total cholesterol (TC, circles), lipoprotein(a) (Lp(a), squares), LDL cholesterol (LDL-C, triangles), and apolipoprotein B (ApoB, diamonds) in days after LDL-apheresis performed at biweekly intervals. B, immediately before apheresis; A, immediately after apheresis

Lp(a) was be measured by a specific radio-immunoassay [apolipoprotein(a) RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden]. Vitamin E (α-tocopherol) concentrations in LDL were measured by high-performance liquid chromatography (HPLC Spectra Physics model 8800, Breda, The Netherlands) [24]. Fatty acid measurements in LDL were performed by gas chromatography (Varian 3400 GC, Houten, The Netherlands) [26].

Statistical analysis

Analyses were performed with procedures available in SPSS (SPSS Inc., Chicago, IL.). Multivariate ANOVA (with correction for repeated measures) followed by *t*-tests were used for normally distributed data, whereas Friedman's ANOVA (with correction for multiple testing) followed by Wilcoxon's signed rank tests were used for differences in means of not normally distributed data. For measurements of agreement the Pearson product-moment correlation coefficient was used. Stepwise least-squares multivariate regression analysis was performed using a maximal p-value in the F-test of 0.05 before a variable could be added and a minimal tolerance (collinearity) of 0.01. A two-sided *P*-value of less than 0.05 was considered to be significant. Results are expressed as means ± SD, unless otherwise indicated.

Results

Plasma lipids and lipoproteins

Due to ongoing treatment with LDL-apheresis and simvastatin baseline lipid and lipoprotein levels were reduced to the pretreatment levels (C_{MAX}) as shown in Table 1. Mean acute reduction of total cholesterol, LDL cholesterol, apo B, Lp(a), and triglycerides were 61, 78%, 76%, 72%, and 68%, respectively ($P<0.001$). HDL cholesterol levels were not influenced by the treatment. After apheresis, LDL carried less cholesterol, as indicated by the LDL cholesterol to apo B ratio, changing from 0.72±0.16 to 0.63±0.15 ($P=0.04$) immediately post-treatment and to 0.44±0.15 ($P<0.001$) on day 1 (Table 2). The rebound after LDL-apheresis of the various lipoproteins was different. Triglycerides recovered rapidly, reaching 83% of the pretreatment level within 1 day after treatment (data not shown). The rebound of total cholesterol, LDL cholesterol, apo B, and Lp(a) was much slower: 50% recovery within 2-4 days, followed by a successive slower rise (Fig.1). The ratio LDL cholesterol to apo B gradually increased and was comparable to the pretreatment value at day 7 (Table 2).

In vitro susceptibility of LDL to oxidative modification

For each subject, LDL was isolated immediately before and after apheresis, and on days

Table 2. Change in chemical composition in percentage of dry mass of low density lipoprotein (LDL) particles ($1.019 < d < 1.063$ g/mL) and LDL cholesterol to apolipoprotein B ratio presented in days after LDL-apheresis

	day 0-B†	day 0-A†	day 1	day 3	day 7	day 10
%FC	8.3±1.0	8.1±0.7	7.4±1.5*	8.2±0.8	8.2±0.7	8.3±0.8
%CE	39.2±3.2	31.1±5.7¶	29.5±3.4¶	37.9±1.9*	40.1±2.5	40.8±2.0
%TG	4.9±1.2	7.9±2.1¶	7.6±2.1¶	6.0±1.1#	5.2±1.1	4.9±1.0
%PL	19.7±2.0	14.1±5.5#	18.5±2.6	19.3±2.3	19.5±1.3	19.5±1.7
%Prot	27.2±4.1	38.8±7.8¶	37.1±6.5¶	28.6±2.9	27.0±3.2	26.6±2.5
C/Prot	1.2±0.3	0.7±0.2¶	0.7±0.2¶	1.1±0.1*	1.2±0.2	1.2±0.2
LDL-C /apoB	0.72±0.16	0.63±0.15*	0.44±0.15¶	0.61±0.16¶	0.68±0.15	0.72±0.17

*Data represent means (±SD); †, day 0 immediately before (B) and after (A) LDL-apheresis; FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid; Prot, protein; C/Prot, cholesterol (FC + cholesterol moiety of CE [≈ 0.59 x weight of CE]) to protein ratio; LDL-C/apoB, LDL cholesterol to apolipoprotein B ratio. Differences versus before apheresis *0.01<P<0.05; #0.001<P<0.01; ¶P<0.001 (multivariate ANOVA, followed by paired t-test)*

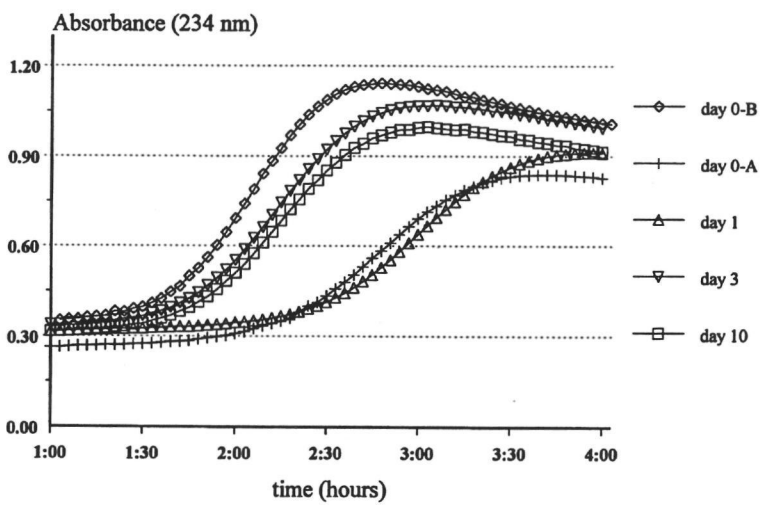

Fig. 2. Example of curve plot depicting the kinetics of LDL-oxidation in one subject immediately before (day 0-B), immediately after (day 0-A), and 1, 3, and 10 days after LDL-apheresis

Table 3. Change in characteristics of the absorbance curves at 234 nm determined by measuring the formation of conjugated dienes after copper-induced oxidation of low density lipoproteins (LDL) in days after LDL-apheresis

	day 0-B†	day 0-A†	day 1	day 3	day 10
lag time (min)	112.3±27.2	132.2±32.1#	130.2±26.4#	116.4±30.2	106.5±26.1
rate (nmol/min/ mg LDL)	11.1±2.7	9.9±4.1	9.1±2.1#	10.9±2.4	11.5±2.2
dienes prod‡ (nmol/mg LDL)	558.2±70.7	497.8±99.6*	500.2±78.9*	555.8±86.5	557.0±48.4
t_{MAX} (min)	185.7±38.8	216.2±47.7#	209.4±34.7#	190.9±41.0	177.6±34.5

*Data represent means (±SD) from duplicate measurements in each patient; †, day 0 immediately before (B) and after (A) LDL-apheresis; ‡, dienes prod, $diene_{MAX}$ minus $diene_{O}$, for definitions see 'Materials and Methods' section. Differences versus before apheresis: *0.01 < P < 0.05; #0.001 < P < 0.01 (multivariate ANOVA, followed by paired t-test)*

1, 3 and 10 post-apheresis. An example of the continuously monitored formation of conjugated dienes, as a measure of Cu^{2+}-induced LDL-oxidation, is shown in Figure 2. The means of the characteristics of the absorbance curves, depicting changes in LDL-oxidizability are shown in Table 3. LDL appeared less susceptible to in vitro oxidation immediately after apheresis and on day 1 post-treatment: all indices for LDL-oxidizability were significantly different from pretreatment. At 3 days post-treatment the indices were again comparable to pre-apheresis and remained unchanged on day 10.

Changes in composition of lipoproteins

The chemical composition in percentage of dry mass was determined immediately before and immediately after apheresis, and on days 1, 3, 7, and 10 post-treatment. Until and including the third day a significant reduction in cholesterol content (predominantly cholesteryl ester) of LDL particles was observed, associated with a relative enrichment in triglycerides and proteins (Table 2). As a consequence, the ratio cholesterol to protein was significantly reduced through the third day post-apheresis, indicating smaller, less buoyant lipoprotein particles. VLDL and IDL particles showed comparable changes as LDLs, which lasted until the first day post-treatment (data not shown).

The fatty acid composition expressed in proportions of total LDL fatty acid content was measured immediately before and immediately after apheresis, and on days 1, 3, and 10 post-apheresis. In general, the fatty acid composition of each isolated LDL showed a decreased amount of unsaturated fatty acids and an increasing percentage of saturated fatty acids (Table 4). The arachidonic acid (20:4) and linoleic acid (18:2) content showed a significant decrease through the first day after apheresis at the ex-

Table 4. Change in fatty acid composition in percentage of low density lipoprotein (LDL) total fatty acid profile in days after LDL-apheresis

	day 0-B†	day 0-A†	day 1	day 3	day 10
Arachidonic acid (20:4)	9.5±3.8	8.8±3.5	7.8±2.2#	9.5±3.2	9.8±3.8
Linoleic acid (18:2)	40.3±4.0	38.7±6.0	35.9±5.3#	40.1±4.4	41.5±5.3
Oleic acid (18:1)	19.7±2.3	18.7±3.6	20.6±4.3	20.2±2.4	19.1±3.3
Stearic acid (18:0)	8.6±1.2	9.8±1.5*	9.2±2.1	8.0±1.2	8.4±1.2
Palmitic acid (16:0)	22.0±5.4	24.0±5.0	26.4±3.6*	22.2±4.0	21.2±4.5

*Data represent means (±SD) in percentages of LDL total fatty acid profile; †, day 0 immediately before (B) and after (A) apheresis. Differences versus pretreatment levels: *0.01<P<0.05; #0.001<P<0.01 (Friedman's ANOVA, followed by Wilcoxon's signed rank test)*

pense of an increase in palmitic acid (16:0) and stearic acid (18:0). No significant changes were observed in the oleic acid (18:1) content.

The absolute vitamin E levels decreased significantly after LDL-apheresis, comparable to the reduction in lipoprotein concentrations. However, when expressed in milligrams per gram of LDL protein or per micromol of linoleic acid no significant reductions in vitamin E levels were observed compared to pretreament levels (Table 5).

Correlations

No correlations were observed between vitamin E, expressed per gram of LDL protein, and any of the lipids and lipoproteins. Difference between pretreatment and immediate post-treatment levels of vitamin E, expressed in mg/L, showed significant correlations with changes in total cholesterol, triglycerides, LDL cholesterol, and apo B, indicating a relation with the reduced number of circulating lipoprotein particles. Using stepwise multivariate regression absolute vitamin E levels were only explained by absolute changes in total cholesterol concentration (variance R^2=0.42, P=0.02). Stepwise addition of other variables did not further improve the explained variance significantly.

No significant correlations were found between the change in any of the oxidizability parameters and change in lipids, lipoproteins, vitamin E, or the chemical composition of LDL or VLDL. Good correlations were observed between the change in all oxidizability indices and the change from pretreatment to the first day post-apheresis

Table 5. Vitamin E content of low density lipoproteins (LDLs) in days after LDL-apheresis

	day 0-B†	day 0-A†	day 1	day 3	day 10
Vitamin E (mg/L)	11.3±2.0	4.6±0.9¶	7.0±1.2¶	8.9±1.5#	10.3±1.4
Vitamin E‡ (mg/g protein)	4.4±1.5	3.8±2.1	4.3±2.0	5.1±1.1§	4.9±1.3§
Vitamin E (mg/μmol linoleic acid)	6.99±1.45	6.11±1.87	6.93±1.52	6.99±0.97	7.37±1.98

Data represent means (±SD); †, day 0, immediately before (B) and after (A) apheresis; ‡, normal range for healthy volunteers: 4.63±1.38 mg/g LDL protein (n=28). Differences versus pretreatment levels: #0.001<P<0.01; ¶P<0.001; differences versus post-treatment levels: §0.01<P<0.05 (multivariate ANOVA, followed by paired t-test)

in palmitic and arachidonic acid content (data not shown). By multiple regression the change in lag time was explained by the change in arachidonic acid and linoleic acid (total variance R^2=0.71, P=0.004), change in the maximal rate of oxidation was explained by the change in palmitic acid (variance R^2=0.49, P=0.01), and change in time to maximal absorbance by the change in arachidonic acid and linoleic acid (total variance R^2=0.66, P=0.008); stepwise addition of other variables did not further improve the explained variance significantly.

Discussion

In the present study we were able to investigate patients with a primary hypercholesterolemia, who were on long-term LDL-apheresis and were treated concomitantly with the HMG-CoA reductase inhibitor simvastatin. The intermittent nature of LDL-apheresis, which results in plasma concentrations above the time-averaged levels for approximately 9 out of 14 days between consecutive fortnightly procedures, has been used as a less favourable aspect of this procedure [5]. In the present study we observed quantitative changes in lipids and lipoproteins which were in accordance with previous data [3-5]. Characteristically, triglycerides returned quickly to the pretreatment levels, within 1 to 2 days, due to fast increase in VLDL synthesis, whereas LDL cholesterol showed a much slower rebound [10]. As a consequence of the apheresis-induced marked reductions in pool size, vitamin E levels were reduced considerably. Nevertheless, short-term favourable effects on LDL-oxidizability were observed in the days post-apheresis, which were explained by significant compositional changes of VLDL and LDL, i.e. reductions of the unsaturated fatty acid content of LDL.

A potential shortcoming of this study may be the concomitant use of simvastatin. This HMG-CoA reductase inhibitor was administered to reduce the post-treatment rebound and to prolong the interval between apheresis procedures [8-10]. It has been shown that statins selectively reduce the concentration of cholesteryl-ester-enriched apo B-containing lipoproteins and consequently decrease LDL-oxidizability [36-38]. Since simvastatin was administered for a longer period before the start of this study and was not discontinued, the observed changes in this study will be evoked by apheresis.

This study did not investigate the long-term effects of LDL-apheresis on oxidizability. The main issue studied here were the changes between two procedures. We found that the acute reduction in cholesterol pool size was accompanied by qualitative changes is the LDL particle, rendering it less susceptible to in vitro oxidative modification. The oxidizability indices showed a longer lag time, indicating more resistance against oxidative damage, and both the rate of oxidation and the amount of dienes formed were significantly lower post-apheresis, as measures of the extent of oxidative modification. Vitamin E, quantitatively the most important lipophilic antioxidant in LDL [22], did not change significantly when expressed per LDL particle or per micromol of linoleic acid. Indeed, a relation between the vitamin E content and resistance against oxidation of LDL has not been demonstrated, unless vitamin E was supplemented [25,39,40].

Moreover, the time span along which changes reversed was rather short, and so the biological consequences of this period of reduced susceptibility to oxidation remains uncertain. Therefore, the observation that there was no increase or overshoot in oxidizability after an initial period with less susceptibility may be of more importance. In trying to explain the reduced oxidizability we considered the physicochemical properties of LDL. On the average, the LDL mass comprises approximately 40% cholesteryl esters, 25% proteins, 20% phospholipids, 10% unesterified cholesterol, and 5% triglycerides [41]. We observed a reduction of the cholesteryl ester content of LDL, yielding less substrate per particle available for oxidation, which was in line with the reduced ratio cholesterol to protein (Table 2) [36]. The fatty acid composition is another important determinant of LDL-oxidizability [22,25,26]. The rate and extent of diene formation are governed mainly by the amount of polyunsaturated fatty acids available for oxidation. The more unsaturated the fatty acids, the more susceptible they are to oxidation [42,43]. The main polyunsaturated fatty acid in LDL was linoleic acid (Table 4). The relative amount of arachidonic acid and linoleic acid were reduced by 18 and 11%, respectively, explaining the decreased susceptibility to oxidation by multiple regression [26].

Reportedly, a reduced susceptibility to oxidative modification can also be found in structural changes in the surface lipid monolayer of the lipoprotein particles by differences in physical state of the LDL particle core [28,44,45]. By analysis of subclasses of LDL, it has been shown that mean particle diameter, molecular weight, and lipid to

protein ratio decrease progressively with increasing density of the particles [46]. In various reports, these small and dense LDLs have been associated with clinical and angiographic indices of coronary artery disease, related to their enrichment with cholesteryl esters and an increased content of cholesteryl oleate compared to cholesteryl linolate [38,44,45,47]. In addition, these small, dense LDL subfractions are more susceptible to Cu^{2+}-induced oxidative modification [45], as has been reported first by our lab [24]. In the present study, lipoprotein particles were also smaller and more dense after apheresis, indicated by the decreased cholesterol to protein ratio. However, they contained less cholesteryl ester and relatively more surface lipid components, so they had a more or less "loose fitting" surface layer (Table 2). This observation concords with the observed change in immunoreactivity against different apo B epitopes of post-apheresis LDL, as has been shown by Gandjini et al [31]. These and other authors argued that newly formed LDL, either derived from VLDL or directly secreted by the liver, are responsible for the compositional changes found after LDL-apheresis [29,31]. The latter authors also showed that post-apheresis LDL did not originate from selective binding of various LDL subfractions to dextran sulfate cellulose beads, by showing equal affinity of per- and post-apheresis LDL for the Liposorber® columns. Our data fit rather well with the assumption of the presence of newly formed post-apheresis LDL. Moreover, it also explains the reduced susceptibility to Cu^{2+}-induced oxidizability in newly formed LDL in comparison to structurally and compositionally different mature LDL.

The present study showed features that are not unique for LDL-apheresis and can be explained on the basis of differences in chemical composition and fatty acid content and differences in structure of the LDL particle [45,48]. It has been shown that treatment with various lipid lowering agents not only results in a decrease in the number of circulating atherogenic lipoprotein particles but also affects the composition and oxidizability of LDL [36,38,45,49]. In conclusion, our data clearly indicated that apheresis-induced fluctuations in LDL cholesterol and vitamin E levels did not influence oxidizability in a negative sense, even on the days LDL cholesterol level was above the time-averaged concentration. In contrast, short-term, decreased susceptibility to in vitro Cu^{2+}-induced LDL-oxidation was observed caused by changes in fatty acid composition in less buoyant LDL particles.

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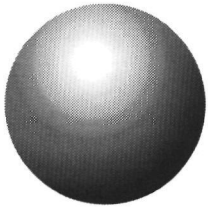

Chapter

S i x

The LDL-Apheresis Atherosclerosis Regression Study (LAARS). Effect of aggressive versus conventional lipid lowering treatment on coronary atherosclerosis

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The LDL-Apheresis Atherosclerosis Regression Study (LAARS). Effect of aggressive versus conventional lipid lowering treatment on coronary atherosclerosis.

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Background. Intensive lipid lowering may retard the progression of coronary atherosclerosis. Low density lipoprotein (LDL) apheresis has the potential to decrease LDL cholesterol to very low levels. To assess the effect of more aggressive lipid lowering with LDL-apheresis, we set up a randomized study in men with hypercholesterolemia and severe coronary atherosclerosis.
Methods and Results. For two years 42 men were treated with either biweekly LDL-apheresis plus medication or medication alone. In both groups a dose of simvastatin of 40 mg per day was administered. Baseline (mean ± SD) LDL cholesterol was 7.8±1.9 $mmol.L^{-1}$ and 7.9±2.3 $mmol.L^{-1}$ in the apheresis and medication group, respectively. The mean reduction in LDL cholesterol was 63% (to 3.0 $mmol.L^{-1}$) and 47% (to 4.1 $mmol.L^{-1}$), respectively. Primary quantitative coronary angiographic endpoints were changes in average Mean Segment Diameter (MSD) and Minimal Obstruction Diameter (MOD). No differences between the apheresis vs medication group were found in MSD (-0.01±0.16 mm vs 0.03±0.16 mm, respectively) or in MOD (-0.01±0.13 mm vs 0.01±0.11 mm, respectively), expressed as means per patient. On the basis of coronary segment, mean percent stenosis of all lesions showed a tendency to decrease; only in the apheresis group more minor lesions disappeared in comparison to the medication group. On bicycle exercise tests the time to 0.1 mV ST-segment depression increased significantly by 39% and the maximum level of ST-depression decreased significantly by 0.07 mV in the apheresis group versus no changes in the medication group.
Conclusions. Two years of lipid lowering both with medication alone or LDL-apheresis with medication showed angiographic arrest of the progression of coronary artery disease. However, more aggressive treatment induced functional improvement, which may precede anatomic changes.

Introduction

The relation between total cholesterol and low density lipoprotein (LDL) cholesterol levels and the incidence of coronary artery disease (CAD) is well established [1,2]. Primary and secondary prevention trials, predominantly conducted in men with hypercholesterolemia, have shown that lipid lowering regimens result in less progression of angiographic lesions [3]. Regression of coronary atherosclerosis is demonstrated to a limited extent in some patients in most of these trials [4-12]. The common denominator of these trials is reduction of LDL cholesterol [13]. Until today intensive lipid lowering in men with established CAD using HMG-CoA reductase inhibitors is the most effective means in terms of slowing or arrest of progression of coronary atherosclerosis [14,15] and consequently reducing the number of clinical events [16].

Continuous LDL-apheresis, using dextran sulfate cellulose columns, selectively removes apolipoprotein B-containing lipoproteins from plasma [17,18]. The performance of regular apheresis permits the achievement of lower levels of LDL cholesterol which is not usually possible to attain with drug therapy alone. The application of this method may offer opportunities in the prevention of progression, or even inducing regression of coronary atherosclerosis in selected patients with a primary hyperlipidemia and established CAD [19-22].

The quantitative computerized analysis of the extent of atherosclerosis on coronary angiograms (QCA) has been developed and extensively evaluated for angiographic trials [23-25]. Despite certain limitations, QCA is one of the most precise procedures available for assessing progression or regression of CAD [26]. Given the relatively small changes in the severity of lesions demonstrated in angiographic trials and the unclear clinical benefits of such changes, the addition of measurements to predict the functional significance of changes in coronary stenosis seems important [27].

The LDL-Apheresis Atherosclerosis Regression Study (LAARS) was designed as a prospective, open, randomized, single-centre study in men with primary hypercholesterolemia and extensive coronary artery disease. The objective was to determine whether more aggressive LDL cholesterol lowering, with biweekly LDL-apheresis plus the HMG-CoA reductase inhibitor simvastatin, exerts better an anti-atherosclerotic effect than lipid lowering to more conventional cholesterol levels with simvastatin alone. In this article the results of sequential exercise tolerance tests and quantitative computer-assisted analysis of coronary angiograms during 2 years of treatment are described and related to the lipid and lipoproteins levels. The results of functional measurements of coronary blood flow by means of videodensitometry will be presented separately.

Patients and Methods

Subjects and treatment

From January 1990 until May 1992, men aged between 30 and 67 years, who underwent diagnostic coronary angiography for angina pectoris were screened for eligibility. Included were patients with a mean of two successive serum total cholesterol determinations above 8.0 mmol.L^{-1} or LDL cholesterol above 5.8 mmol.L^{-1}, and a mean of two successive fasting serum triglycerides measurements below 5.0 mmol.L^{-1} on a standard lipid lowering diet without other lipid lowering treatments, and extensive coronary atherosclerosis as shown by visual assessment of their coronary angiogram. Excluded were patients with a ventricular ejection fraction <0.35, acute myocardial infarction or percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG) within the previous 3 months, impaired hepatic (>30% above normal range) or renal function (plasma creatine ≥150 μmol.L^{-1}), hypertension (diastolic blood pressure ≥100 mmHg), diabetes mellitus, severe obesity (BMI ≥30 kg.m^{-2}), homozygous familial hypercholesterolemia, any secondary hyperlipidemia, and heavy smokers (>10 cigarettes per day). Patients with a history of PTCA and CABG were included because these modalities have become an integral part in the treatment of patients with CAD. Excluding these patients would have introduced a selection bias. Both patients with and without PTCA or CABG had severe coronary atherosclerosis till the same degree (Table 1).

At least 2 months prior to the start of the study all lipid lowering drugs were stopped. Only a cholesterol lowering diet equivalent to the American Heart Association step I diet was continued or prescribed and the patients were instructed by a dietician. During this 2-months run-in period an exercise test and coronary angiography were performed. Written informed consent according to the Declaration of Helsinki was obtained. Those patients who met all in- and exclusion criteria were allocated at random to either biweekly LDL-apheresis plus simvastatin (40 mg/day), a potent HMG-CoA reductase inhibitor [28,29], or simvastatin treatment (40 mg/day) alone. Randomization was stratified for the level of serum total cholesterol and lipoprotein(a) [Lp(a)], age, and CABG status.

LDL-apheresis was performed with an automated system with two small-sized dextran sulfate cellulose columns (MA-01 unit, Kanegafuchi Chemical Industry Co. Ltd., Osaka, Japan). In this system the plasma is separated by a polysulfone membrane separator, and apolipoprotein B-containing particles are adsorbed in one of two columns containing cellulose-bound dextran sulfate used in rotation in a veno-venous extracorporeal circuit. A volume of 5000 mL (approximately 1.5 plasma volume) was treated per session. The combination therapy of LDL-apheresis and simvastatin can be expected to slow down the post-apheresis rebound in serum cholesterol, which permits prolongation of the intervals between the apheresis procedures [30,31]. Patients allo-

cated to lipid lowering with drugs attended the outpatients clinic each month; those on LDL-apheresis were seen fortnightly. In both groups a resin in the highest tolerable doses was added to the treatment if (pre-apheresis) serum cholesterol levels in 2 consecutive months remained above 8.0 mmol.L^{-1}, since it was considered not appropriate to continue single drug treatment above this level. At each visit, the patients were subjected to a brief physical examination, and additional dietary instruction was repeated frequently. Antianginal medication was continued at the same doses during the study. If adaptation was necessary during the study, the original prescription, if possible, was restored before the follow-up heart catheterization.

Lipids, lipoproteins and laboratory safety measures

In both groups lipids, lipoproteins, blood chemistry, hemograms, and routine urinalysis were performed monthly. Apolipoprotein (apo) A1, apo B, and Lp(a) were measured bimonthly. In the apheresis group lipids and lipoproteins and some extra laboratory safety parameters (hemogram, calcium, and total protein level) were measured before and immediately after each LDL-apheresis. Serum total cholesterol and fasting triglycerides were determined enzymatically (CHOD-PAP, no. 237574, Boehringer Mannheim GmbH, FRG and Sera-PAK, no. 6639, Miles, Italy, respectively). High density lipoprotein (HDL) cholesterol was determined using the polyethylene glycol 6000 precipitation method [32]. LDL cholesterol was calculated by subtraction. Samples for apo A1, apo B, and Lp(a) were stored at -80°C, and determined at the end of the study. Apo A1 and apo B were quantified in serum by immunonephelometry [33]. Lp(a) was measured by a specific radioimmunoassay (apolipoprotein(a) RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden). Hyperhomocysteinemia was excluded by measuring fasting homocysteine levels [34]. Fibrinogen levels did not differ between both groups. Apheresis produced an acute 35% reduction of fibrinogen, returning to pretreatment levels between 2 and 7 days (n=11, data not shown).

The selective removal of apo B-containing lipoproteins with LDL apheresis causes sawtooth-like alterations in lipoprotein concentrations [35]. The increase of lipoprotein levels after the treatment can be explained by first-order kinetics [36-38]. Therefore, time-averaged concentrations (C_{AVG}) or interval means of total cholesterol, LDL cholesterol, apo B, and Lp(a) were calculated by applying a formula derived from the rebound curves which were constructed for each patient at one occasion: $C_{AVG}=C_{MIN}+0.73(C_{MAX}-C_{MIN})$, where C_{MAX} is the pretreatment level and C_{MIN} the levels immediately after apheresis [39]. For serum triglycerides and HDL cholesterol only pretreatment levels were used in the analysis because triglycerides reach pretreatment levels within 1 to 2 days after apheresis and HDL cholesterol is not influenced by LDL-apheresis.

Exercise tests

Bicycle exercise tests were done at baseline, after 12 months, and after 24 months of the start of the study. The assessments at the end of the study were done 3 to 4 weeks after the last apheresis. An electronic braked ergometer (Marquette Case 15, Marquette Electronics Inc., Milwaukee, USA) was used, starting at a load of 50 W and raising it every minute by 10 Watt during continuous ECG monitoring to maximum exercise limited by chest discomfort or usual criteria for stopping the test. Blood pressure and 12-lead ECG registration were monitored at rest, at maximum exercise, and every minute during the test. Automated calculation of ST-segment depression was performed at a point located 80 msec beyond the J-point. Data were corrected for systolic blood pressure-heart rate product at maximal load. An additional exercise thallium scintigraphy was performed when the bicycle exercise test was not conclusive due to a maximum heart frequency $\leq$85% of the predicted value corrected for age and body mass, and/or the predicted load $\leq$80% without ST-segment changes.

Coronary angiography

The coronary angiograms were obtained at baseline and after 2 years of treatment, 4 weeks after the last apheresis, using the same protocol as has been described for the Regression Growth Evaluation Statin Study (REGRESS) [12]. During both procedures the same non-ionic iso-osmolar contrast agent [Iohexol 350 (Nycomed AS, Oslo, Norway)] and the same cineangiographic techniques were used, according to standard requirements for quantitative analysis [25]. A centimeter grid was filmed to adjust for pincushion distortion. The same type and diameter of catheters were used in both procedures and used as a scaling device in QCA analysis. The protocol required administration of 5 to 10 mg of isosorbidedinitrate sublingually 5 minutes prior to the first intracoronary injection of contrast, which was repeated during the procedure if necessary. Twelve to 15 coronary segments were filmed in 2 projections [40]. Preferably, end-diastolic frames were selected for blinded computer-assisted quantitative analysis of paired angiograms, which was performed at the Heart Core Angiographic Reference Laboratory at the University Hospital of Leiden using the Cardiovascular Measurement System (CMS-Medis Medical Imaging Systems, Nuenen, the Netherlands [CMS-version 2.3D]) [41]. This system uses a high-quality cine-video converter (CAP 35E) that allows a selected cine frame to be projected onto a digital camera through a zoom lens (magnification 2.3x). The video signal of the magnified region was digitized at a matrix size of 512x512x8 bits. For calibration, the boundaries of a nontapering part of the catheter were determined automatically over a length of approximately 2 cm. To assess the contours of the vessel, the beginning and the end of the specific coronary segment had to be indicated, after which a pathline was computed connecting these two points. The contours of the vessel were then computed in multiple iterations by the minimal cost contour detection technique. The edge strength of a point was based on

the weighted sum of the first and second derivative functions; this edge strength was corrected for the limited resolution of the entire imaging chain, a procedure that is particularly important for the accurate measurement of small vessels. A diameter function was determined in absolute terms (in mm) by computing the shortest distances between the left and right contours along the vessel centerline. The reference diameter was defined as previously described [42]. Primarily, minimal obstruction diameter (MOD), as a measure for localized atherosclerosis, and the mean segment diameter (MSD), as a measure for diffuse changes, were assessed. Only segments without overlap and minimal foreshortening were analyzed, including bypass segments. PTCA segments from procedures after the randomization and segments distal to an occlusion were excluded from analysis. Patients were categorized with regard to MOD and clinical events as regressors, stable patients, and progressors, according to the REGRESS protocol [12]. Patients with at least one lesion worsening by ≥0.4 mm or development of a lesion which reduced the lumen diameter by ≥0.4 mm were defined as progressors. Regressors were patients with at least one lesion improving ≥0.4 mm and no lesions worsening ≥0.4 mm. Stable patients had no lesions worsening or improving by ≥0.4 mm. Patients with regressing and progressing lesions were considered to be progressors because simultaneous progression and regression reflect an unstable process in coronary atherosclerosis [10]. If a patient had suffered from a myocardial infarction or unstable angina, he was considered to be a progressor irrespective of angiographic outcome. Additionally, the percentage of stenoses in each segment was calculated as the mean of the percent stenosis in 2 projections, assuring for comparability of the segments by available angiographic landmarks, in the baseline and in the follow-up angiograms. Only segments with a mean percentage of stenosis ≥20% at either baseline or follow-up were analyzed. If only one projection was available of a stenosis ≥20%, this figure was used as the mean percent stenosis in that particular angiogram. New lesions were defined as <20% at baseline and ≥20% at follow-up.

Statistical analysis

The sample size was limited for logistic reasons to approximately 40 patients. At the start of the study the observed SD for mean progression in coronary segments from previous trials was 0.23 to 0.32 mm (0.32 mm with baseline total cholesterol >6.0 $mmol.L^{-1}$). Considering the expected changes in cholesterol levels a minimal sample size of 19 patients in each group was calculated (SD=0.25 mm, expected difference=0.20 mm) [25].

Analyses were done with procedures available in the statistical package for social sciences (SPSS Inc., Chicago, IL.), using Student's *t*-test and multivariate ANOVA (with correction for repeated measures or covariates) for normally distributed data, or Mann-Whitney U-test for differences in means of not normally distributed data. Differences in proportions were analyzed with the (Yates') corrected chi^2-test, and analyses

for trends in proportions were performed with the extended Mantel-Haenszel chi^2-test. A two-sided Fisher's exact test was used when the total number of cases was less than 15. For measures of agreement the Pearson product-moment correlation coefficient was used. Analyses were based on randomization assignment, except for one patient in the apheresis group, who died within 3 months from the start of the study. A two-sided P-value of less than 0.05 was considered significant. Results are expressed as means ± SD, unless otherwise indicated.

Table 1. Baseline characteristics (numbers (percentages) or means ± SD)

	Apheresis (n=21)	Medication only (n=21)	P*
Age [years]	50.2±9.6	53.9±8.7	0.43
Weight [kg]	81.5±9.7	80.8±8.6	0.88
BMI [kg.m^{-2}]	26.6±2.0	26.2±2.0	0.64
Blood pressure [mmHg]			
systolic	129.3±17.3	126.3±18.1	0.56
diastolic	78.2±8.9	76.5±9.0	0.63
Current smoking	3 (14.3)	4 (19.0)	1.00
Vascular disease			
Infarction	16 (76.2)	18 (85.7)	0.69
CABG	10 (47.6)	10 (47.6)	0.75
PTCA	2 (9.5)	5 (23.8)	0.41
Hypertension	2 (9.5)	5 (23.8)	0.41
Stroke	1 (4.8)	3 (14.3)	0.60
Claudication	3 (14.3)	5 (23.8)	0.69
Drug treatment			
ß-Blocker	10 (47.6)	14 (66.7)	0.35
Ca-channel blockers	4 (19.0)	7 (33.3)	0.48
Long acting nitrates	4 (19.0)	4 (19.0)	0.69
Anticoagulants	3 (14.3)	6 (28.6)	0.45
Platelet aggregation inhibitors	4 (19.0)	8 (38.1)	0.31

**t-test or chi^2-test where appropriate. BMI, body mass index; CABG, coronary artery bypass graft; PTCA, percutaneous transluminal coronary angioplasty*

Results

Baseline characteristics

In both treatment groups 21 men were enrolled of whom baseline characteristics are shown in Table 1. In both groups, 16 patients were heterozygous for familial hypercholesterolemia (76% of the study population). Risk factors for atherosclerosis were equally distributed. All patients had severe coronary atherosclerosis. A previous history of myocardial infarction was present in 16 versus 18 men in the apheresis and the medication group, respectively, and CABG had been performed in 10 men in both groups. By the criterion of a stenosis of ≥50% being considered significant, 17 of 21 men in the apheresis group, and 19 of 21 men in the medication group had 3-vessel disease of the coronary arteries, and the other patients had 2-vessel disease. Drug treatment at randomization showed a higher but not statistically significant number of patients in the medication group using anticoagulants or platelet aggregation inhibitors (Table 1). At the time the study started anticoagulants or platelet inhibitors were less used. Baseline cholesterol levels were high, and predominant elevation of apo B-containing lipoproteins was found in agreement with the inclusion criteria (Table 2). Lp(a) levels showed a skewed distribution with median baseline levels of 28.8 and 19.8 $mg.dL^{-1}$ in the apheresis and medication group, respectively. The treatment groups were well balanced and no significant differences were found with respect to baseline characteristics and baseline lipid and lipoprotein concentrations.

Clinical events and patient evaluation

Three patients in the apheresis group and 5 in the medication group had to be hospitalized for unstable angina (Table 3). One of these patients in the apheresis group was lost to follow-up, due to death immediately after coronary surgery within 3 months after the start of the study. From the other two patients in the apheresis group with unstable angina one had to undergo CABG at 9 months after the start of the study and continued only treatment with simvastatin after this procedure; the other one had to undergo a PTCA procedure at 12 months and continued treatment with LDL-apheresis afterwards. Four men in the apheresis group had a myocardial infarction, all within 6 months (range 2-6) after the start of the study. In all these patients LDL-apheresis and simvastatin treatment were continued. Unstable angina in the medication group was observed 5-24 months after the start of the study, causing hospitalization and adjustment of antianginal drugs, and 2 interventions (PTCA and CABG) at the end of the study. A total of 7 cardiac events (unstable angina and infarction) in 7 different patients in the apheresis group versus 5 events in 5 different patients in the medication group was observed (P=0.73). Most events took place in the first year of treatment with a median of 5 versus 5.5 months after the start of the study in the apheresis and medication group, respectively. There were no significant differences between both groups (Table 3).

Table 2. Changes in lipids and lipoproteins: baseline and treatment levels (means ± SD)

	Apheresis (n=21)					Medication only (n=21)			Diff
	basal	before	after	interval mean	% change	basal	mean	% change	*P*
Tot chol	9.72±1.84	5.60±1.26	2.06±0.46	4.63±1.18	-52.9±6.6	9.85±2.17	5.95±1.60	-39.5±7.7	0.005
Triglyc	2.32±1.03	1.83±0.76	0.48±0.28	1.83±0.76	-17.4±24.4	2.64±1.33	1.84±0.89	-26.5±20.3	0.38
LDL chol	7.78±1.86	3.72±1.26	0.82±0.41	2.95±1.13	-62.9±8.3	7.85±2.34	4.13±1.58	-47.4±8.1	0.01
HDL chol	0.93±0.18	1.09±0.20	1.08±0.20	1.09±0.20	+17.7±13.6	0.92±0.19	1.05±0.22	+13.7±10.9	0.23
Lp(a)	57.0±63.9	59.1±68.8	13.8±15.3	44.5±54.3	-18.6±18.0	38.4±39.7	44.5±45.7	+14.9±16.3	0.02
Apo A1	1.43±0.29	1.34±0.17	1.08±0.13	1.34±0.17	-5.3±13.0	1.46±0.38	1.33±0.21	-5.4±16.8	0.89
Apo B	2.59±0.47	1.65±0.43	0.45±0.16	1.32±0.35	-49.0±7.6	2.60±0.61	1.74±0.47	-31.0±17.0	0.003

Cholesterol and Triclycerides levels in mmol.L^{-1}, Lp(a) in mg.dL^{-1} and Apo A1 and B in g.L^{-1}. Apheresis group: 52 measurements per patient. Medication group: 26 measurements per patient. Before, pre-treatment levels (C_{MAX}); after, post-treatment levels (C_{MIN}); interval mean, time-averaged levels (C_{AVG}), calculated as follows: $C_{AVG} = C_{MIN} + 0.73(C_{MAX} - C_{MIN})$ (see text); % change, difference between basal and mean levels; P, P-values of differences between interval mean in the apheresis group and mean levels in the medication group (t-test or Mann-Whitney U-test where appropriate). To convert values for total cholesterol to mg/dL, multiply by 38.67, and to convert values for triglycerides to mg/dL, multiply by 88.57

Lipid and lipoprotein profiles

Three patients in the apheresis group and 4 in the medication group received additional resin treatment, 8-24 g cholestyramine per day. LDL-apheresis caused an acute reduction of 62%, 78%, 71%, and 72% of the mean concentrations of total cholesterol, LDL cholesterol, Lp(a) and apo B, respectively (Table 2). HDL cholesterol levels were not influenced by this procedure, and apo A1 levels were acutely decreased on the average by 20%. Pretreatment levels of total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, apo A1, and apo B in the apheresis group were not significantly different in comparison to mean levels in the medication group. Pretreatment levels of Lp(a) in the apheresis group did not change compared with basal concentrations, whereas an increase of 15% (P=0.03) in the medication group was found. Differences in treatment effects were established by comparison of interval mean concentrations in the apheresis group and mean concentrations in the medication group. During the entire course of the study a constant reduction of 63% of LDL cholesterol was found in the apheresis group to an interval mean level of 2.95±1.13 mmol.L^{-1}. Total cholesterol, LDL cholesterol, and apo B showed the same course and were significantly lower in comparison to the medication group (Table 2). HDL cholesterol levels at baseline and during the study were comparable in both groups; an increase of 14 -18% was observed while on treatment with simvastatin (Table 2). Although significantly reduced, the mean serum triglyceride levels showed no differences between the groups. The LDL/HDL choles-

Table 3. Clinical events

	Apheresis	Medication only	P*
Hospitalization for unstable angina	3	5	0.69
of whom PTCA	(1)	(1)	0.47
of whom CABG	(2)†	(1)	1.00
Nonfatal myocardial infarction	4	0	0.11
Death	1	0	1.00
Claudication	2	2	0.60
of whom PTA	(1)	(0)	1.00
of whom bypass surgery	(0)	(1)	1.00
Stroke / TIA	0	1	1.00

*Numbers between brackets represent a subdivision, e.g. 1 of the 3 patient hospitalized for angina had a PTCA. *, chi^2-test; †, one patient died. PT(C)A, percutanous transluminal (coronary) angioplasty; CABG, coronary artery bypass graft surgery; TIA, transient ischemic attack*

Table 4. Bicycle exercise electrocardiography at baseline, after 1 year, and after 2 years of treatment (means±SEM)

	Apheresis (n=17)				Medication only (n=15)				Diff
	Test 1	Test 2	Test 3	P_A	Test 1	Test 2	Test 3	P_M	P_D
Ex-time	687±36	674±43	702±41	0.34	687±65	630±44	667±60	0.19	0.21
ST-time	461±47	548±51	641±50	0.001	485±81	464±54	442±60	0.52	0.001
ST-max	1.4±0.2	0.8±0.2	0.7±0.2	0.000	1.4±0.3	1.3±0.3	1.4±0.4	0.76	0.008
Sbphr-max	29842±2020	29089±1311	29458±1549	0.89	27141±1518	26090±1015	26119±1169	0.60	0.84
Load-max	151±6	152±7	157±7	0.46	154±10	147±7	151±10	0.29	0.30

Test 1, 2, and 3: exercise test at baseline, after 1 year, and after 2 years of treatment, respectively; Ex-time, maximal exercise time (sec); ST-time, time to 1 mm (= 0.1 mV) ST-depression (sec); ST-max, maximal ST-depression (mm); Sbphr-max, systolic blood pressure - heart rate product at maximal exercise (mmHg/min); Load-max, maximal load (W); P_A, P_M and P_D, p-values in Apheresis (A) group, Medication (M) group, and of the difference (D) between Apheresis and Medication group, respectively (multivariate ANOVA)

terol ratio was reduced from 8.4 to 2.7 (-68%) in the apheresis group, versus -54% (from 8.5 to 3.9) in the medication group, respectively. Time-averaged Lp(a) levels were reduced by 19% in the apheresis group, which were significantly different in comparison with increased levels found in the medication group (Table 2).

Exercise tests

Seventeen and 15 pairs of bicycle exercise tests could be evaluated in the apheresis and medication group, respectively (Table 4). The patient from the apheresis group who died early in the study was excluded from analysis. At baseline no differences in exercise tolerance by bicycle tests were found between both groups. After 1 year and after 2 years of treatment a significant increment in time to 0.1 mV ST-depression (ST-time) was observed in the apheresis group, accompanied by a significant decrease of the ST-depression at maximal load (ST-max), whereas no changes were found in the medication group (Figure 1). These differences remained highly significant when corrected for the product of systolic blood pressure and heart rate at maximal exercise and when the patients who had a PTCA, CABG, or myocardial infarction were excluded (data not shown). None of the patients with a negative or inconclusive test showed conversion to

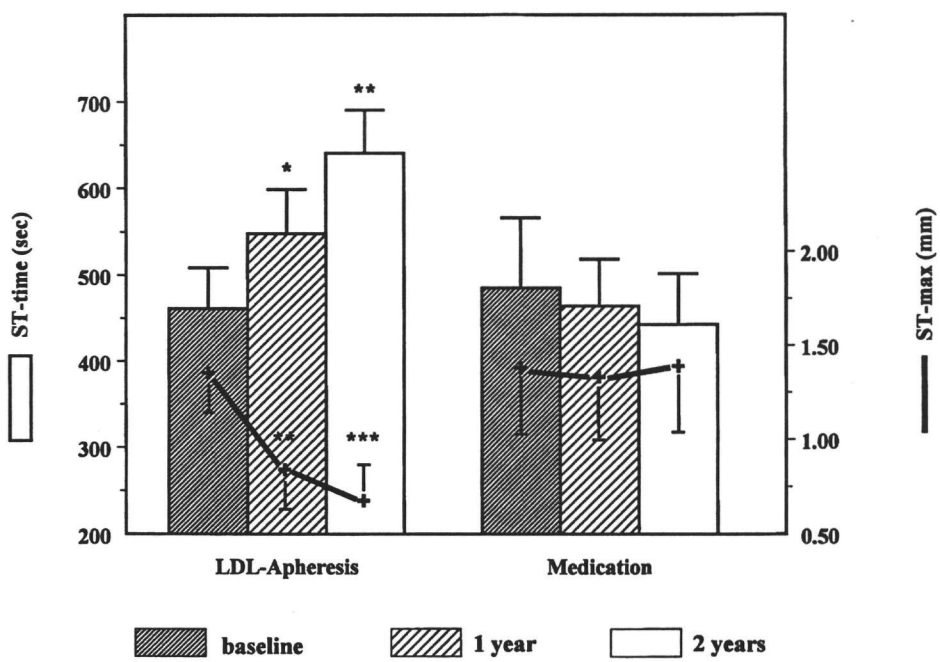

*Fig. 1. Time to 1 mm (=0.1 mV) ST-depression (ST-time in sec) and maximal ST-depression (ST-max in mm) in the LDL-apheresis group (n=17) versus the medication group (n=15) at baseline, after 1, and after 2 years of treatment; asterisks denote significant differences: *0.01≤P<0.05; **0.001≤P<0.01; ***P<0.001 (t-test)*

Table 5. Results of quantitative computer analysis of the coronary angiographies, baseline and at the end of the study (means ± SD)

	Apheresis				Medication only				Diff
	No	baseline	follow-up	change	No	baseline	follow-up	change	*P*
Per patient analysis	20				20				
mean segment diameter		2.65±0.49	2.63±0.41	-0.01±0.16		2.58±0.51	2.60±0.48	0.03±0.16	0.46
min obstruction diameter		1.93±0.43	1.92±0.40	-0.01±0.13		1.89±0.50	1.90±0.46	0.01±0.11	0.54
percent stenosis									
(≥20-50%)		31.3±5.5	31.9±5.8	0.6±2.3		33.2±5.4	33.9±4.4	0.7±3.5	0.65
(>50%)		-	-	-		-	-	-	-
Per segment analysis	173				178				
mean segment diameter		2.59±0.96	2.59±0.95	0.00±0.24		2.56±1.00	2.59±0.97	0.02±0.24	0.29
min obstruction diameter		1.93±0.93	1.92±0.92	-0.01±0.26		1.89±0.98	1.90±0.94	0.01±0.26	0.48
percent stenosis									
(20-50%)	177	30.1±7.6	29.6±11.4	-0.6±8.4	161	32.8±8.6	32.0±11.5	-0.8±9.1	0.82
(>50%)	18	56.0±7.6	52.5±12.4	-3.5±9.5	25	57.0±6.6	52.6±11.8	-4.5±12.3	0.78

Change, difference of follow-up minus baseline; P, p-value of treatment effect comparing changes in the apheresis group with the changes in the medication group (t-test). In the per patient analysis mean percent stenosis >50% were not present

a positive exercise test.

After exclusion of the patients who had a CABG procedure, 2 and 6 paired exercise thallium scintigraphies were available in the apheresis and medication group, respectively. The value of the analysis of the scans is limited due to the small numbers. However, no differences were found in the number of segments with perfusion defects or redistribution within and between both groups (data not shown).

Quantitative analysis of coronary angiography

The coronary angiograms of 20 patients could be evaluated in both groups. The two patients who could not be evaluated at follow-up included one assigned to the apheresis group who underwent CABG surgery and died within 3 months after the start of the study, and one patient assigned to the medication group whose film quality was insufficient for paired analysis. Bypass grafts were included in the analysis, 16 and 22 segments in the apheresis and medication group, respectively. Paired measurements were available on 351 segments, with a mean of 8.8±2.2 (range 4-15) segments analyzed per patient in both groups. No significant differences were found for the MSD and the MOD between and within the groups analyzed on patient or on segment basis (Table 5). The mean change per patient in percent stenosis was not different for both groups. The analysis on a segment basis of the changes in percent stenosis showed a comparable reduction in both minor (20-50%) and major (>50%) stenotic segments in both treatment groups (Table 5). However, in the apheresis group, the total number of lesions was decreased due to the disappearance (<20%) of 40 minor stenoses versus 20 in the medication group (P=0.005), whereas 23 versus 32 new stenoses were found, respectively (P=0.19). By categorical approach 9 patients in the apheresis group and 11 patients in the medication group were classified as progressors. Two and 5 patients were regressors, respectively, and the remaining men showed stable disease.

Correlations

Of baseline variables, total cholesterol (r=-0.51, P=0.01), LDL cholesterol (r=-0.48, P=0.02), ratio LDL/HDL cholesterol (r=-0.63, P=0.001), and apo B (r=-0.49, P=0.01) were correlated with time to 0.1 mV ST-segment depression. Relative changes from baseline of total cholesterol and LDL cholesterol were also significantly correlated with the change in time to 0.1 mV ST-segment depression on the exercise ECG (Table 7). No correlations were found between baseline and in-trial lipid and lipoprotein levels and MSD or percent stenosis. Only mean in-trial concentrations of total cholesterol, LDL cholesterol, ratio LDL/HDL cholesterol, and apo B were associated with the percent change in MOD (Table 7, Figure 2). No correlations were found between time to 0.1 mV ST-segment depression on the exercise ECG and MOD. An association was found between maximal ST-depression on the exercise ECG and the MOD at baseline (r=-0.48, P=0.006) and after 2 years of treatment (r=-0.39, P=0.03).

Table 6. Correlations between lipids and lipoproteins and some outcome variables, expressed as percent change from baseline

	Change in ST-time		Change in MOD	
	r	*P*	r	*P*
Change from baseline				
total cholesterol	0.42	0.04	0.25	0.12
LDL cholesterol	0.52	0.01	0.30	0.06
LDL/HDL ratio	0.39	0.06	0.35	0.03
apolipoprotein B	0.39	0.06	0.22	0.16
Mean in-trial concentrations				
total cholesterol	-0.34	0.10	-0.41	0.009
LDL cholesterol	-0.37	0.08	-0.43	0.006
LDL/HDL ratio	-0.30	0.15	-0.37	0.01
apolipoprotein B	-0.36	0.09	-0.35	0.03

Data are pooled for exercise tests (n=32) and for quantitative coronary angiography (n=40). ST-time, time to 0.1 mV ST-depression on the exercise ECG; MOD, minimal obstruction diameter; r, Pearson's correlation coefficient; P, P-value

Treatment side-effects

No significant differences were found between and within the groups for serum creatinine levels, fasting blood glucose, alkaline phosphatase, and leucocyte counts. The patients on apheresis experienced a significant fall in hemoglobin level from 9.2±0.6 $mmol.L^{-1}$ to 8.6±0.6 $mmol.L^{-1}$ (-6.0±5.8%), due to the procedure. A nonspecific acute loss of 12 % of serum protein levels was observed directly post-apheresis, without trend in change of pretreatment levels. Twelve of 1039 (1.2%) apheretic procedures were complicated by an episode of hypotension (systolic blood pressure ≤80 mm Hg), not leading to discontinuation of the treatment. No bleeding complications were observed the first days after LDL-apheresis. Only 2 sessions in one patient had to be interrupted due to an 'anaphylactoid' reaction caused by the temporary administration of an ACE-inhibitor [43,44]. During the administration of simvastatin no subjective adverse experiences were observed. Episodes with aminotranferase levels >3 times the upper limit

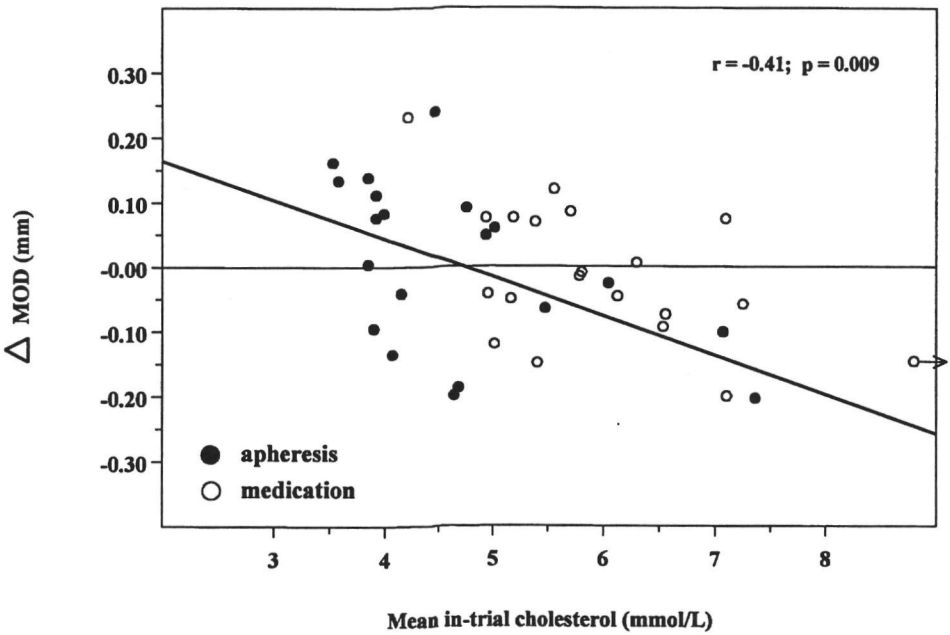

Fig 2. Correlation between mean in-trial total cholesterol concentration and the change in minimal obstruction diameter (MOD) [a negative value depicts progression]. Open symbols represent the medication group and closed symbols the apheresis group

($\leq$30 U.L^{-1}) did not occur: 3 patients in the apheresis group and 1 patient in the medication group had $\geq$50% of the measurements of alanine aminotransferase (ALAT) between 31 and 99 U.L^{-1}. Creatine phosphokinase (CK) levels were elevated in 3 patients in both groups in $\geq$50% of the measurements (range 101-468 U.L^{-1}; upper limit of normal $\leq$100 U.L^{-1}). None of the patients had to discontinue the administration of simvastatin.

Discussion

The LAARS trial was performed to evaluate whether aggressive lipid lowering using LDL-apheresis in men with extensive coronary artery disease, of whom the majority had a familial hypercholesterolemia, will exert better retardation of the progression of coronary atherosclerosis in comparison to conventional treatment. The study showed that the addition of biweekly LDL apheresis to lipid lowering treatment with a HMG-CoA reductase inhibitor improved the ischemic treshhold clearly, whereas an equal effect in angiographically derived measures for coronary atherosclerosis was observed

in both groups, in whom progression of disease would be usual [45,46].

The accepted indication for LDL-apheresis is resistance to drug treatment in patients with CAD [47]. In LAARS LDL-apheresis was used as a method to lower LDL cholesterol more aggressively in subjects of whom some were not drug-resistant. Our results confirm the usefulness of extracorporeal therapy in achieving and maintaining extremely low levels of LDL cholesterol while preserving HDL, with an acceptable safety profile, as has recently been shown [38,47]. The reduction of baseline LDL cholesterol levels of 7.8 $mmol.L^{-1}$ in the apheresis group by 63% is in keeping with other studies using LDL apheresis [22,48,49]. On the other hand, LDL reduction from 7.9 $mmol.L^{-1}$ in the medication group by 47% may be considered as a good response in comparison to recent studies using HMG-CoA reductase inhibitors, which is probably the result of frequent monitoring of our patients [5,8,10,11]. Since LDL apheresis entails a major commitment for the patient and medical community, these latter results stress that only primary hypercholesterolemic patients with established CAD refractory to drug treatment should be treated with LDL-apheresis.

Five prospective trials that include angiographic endpoints in patients with severe hypercholesterolemia using LDL apheresis-containing protocols to lower LDL cholesterol have recently been described [38,48-51]. The FH Regression Study [38] and our study are the only randomized ones. Both studies show no further improvement of the angiographic endpoints by the addition of LDL-apheresis to conventional lipid lowering treatment. However, changes in measures for diffuse atherosclerotic disease (MSD) and focal disease (MOD and % stenosis) in both studies were comparable with pooled data from intervention groups of 5 recent angiographic regression studies [5,7,8,10,11] using HMG-CoA reductase inhibitors, as has been described by Thompson et al [38]. Therefore, intermittent LDL reduction by apheresis induces arrest of progression of angiographically visible lesions comparable to drug treatment.

Although LAARS demonstrated a correlation between the change in MOD and mean in-trial concentrations of LDL, it is not clear why further LDL lowering in the apheresis group did not result in more pronounced mean changes in angiographic outcome measures in comparison to the medication group. In contrast to the FH Regression Study [38] LDL cholesterol levels in the apheresis group from our study were more reduced than in the medication group. In the first study mean LDL cholesterol levels were 3.2 $mmol.L^{-1}$ in the apheresis group versus 3.4 $mmol.L^{-1}$ in the medication group, whereas in our study levels of 3.0 and 4.1 $mmol.L^{-1}$ were reached, respectively. The significance of the observed correlation between MOD and in-trial LDL in our study suggests that the non-sustained reduction of LDL cholesterol due to the rebound after LDL-apheresis does not play a role [38,52]. Sample size and the duration of the intervention may be more important, because expected changes in percent stenosis after a relative short period of intervention have been shown to be too optimistic [14]. Recent data from the Multicentre Anti-Atheroma Study (MAAS) and the Scandinavian

Simvastatin Survival Study (4S) support this view [11,16]. MAAS showed a trend to improvement, but no statistical differences, of the angiographic measures MOD and MSD after 2 years of simvastatin treatment compared to placebo, whereas after 4 years of treatment significant differences were observed angiographically [11]. The 4S trial showed that the effect of simvastatin treatment on coronary events started after 1 year of therapy and increased steadily thereafter [16]. Therefore, it is not unexpected that differences between the apheresis and medication group in LAARS were not angiographically detectable.

The reduction of time-averaged levels of Lp(a) in the apheresis group from LAARS was much less in comparison to LDL. This may be caused by an increase of Lp(a) concentrations associated with the administration of simvastatin which was also found in the medication group, and has been confirmed by others [38,53]. However, an increased rebound of Lp(a) after apheresis in comparison to LDL may also play a role [31,54]. One of the objectives of the FH Regression Study [38] was to verify whether lowering of Lp(a) concentrations by apheresis was associated with further reduction of the percent diameter stenosis of coronary arteries. No benefit could be shown of reducing Lp(a) levels in patients whose LDL cholesterol levels had been effectively lowered by drug therapy or apheresis. Our data confirm these results, particularly because the differences in mean in-trail LDL cholesterol concentrations in the medication and apheresis group were greater than in the FH Regression Study, with comparable changes in Lp(a) levels in both studies. So, the question of the clinical relevance of increased Lp(a) levels during treatment with a HMG-CoA reductase inhibitor seems current.

Natural occurring progression of CAD is mainly seen in the formation of new coronary lesions and less in growth of preexisting ones, and progression of the latter is correlated with high cholesterol level [46]. Most regression studies showed the greatest benefit in atheroma obstructing >50% of the lumen [13], and some reported that mainly smaller lesions responded [9-11,55]. In the present study percent stenosis remained almost unchanged during the 2 years of treatment (97% of stenoses showed changes in degree of <20%), and no preference for changes in severe (>50%) stenoses was observed. It was found, however, that more aggressive lipid lowering with LDL apheresis resulted in the disappearance of more mild to moderate (20-50%) lesions, whereas the formation of new lesions appeared to be comparable between the apheresis and medication group. Therefore, our data seem to be in favor of reducing more early lesions as a response to aggressive lipid lowering of relatively short duration, as has also been shown in the Canadian Coronary Atherosclerosis Intervention Study (CCAIT) and MAAS [10,11]. This may be important, since early lipid-rich lesions with a fine fibrous cap are prone to rupture and lead to thrombotic occlusions and consequent clinical events [56]. Therefore, more aggressive lipid lowering seems to improve stabilization and regression of these lesions.

A remarkable observation of our study was the improvement in ergometric bicycle tests. This has also been found in some uncontrolled studies while applying LDL-apheresis, and may be present within weeks from the start of treatment [50,51]. We observed significant improvement of the exercise tests after 1 year of treatment, which further increased after 2 years. Changes in the time to 0.1 mV ST-depression after 2 year of treatment were also significantly correlated with the amount of LDL reduction. These findings and the angiographic ones suggest that mechanisms other than changes in stenosis play a role in the outcome of the exercise tests. Indeed, cholesterol lowering with HMG-CoA reductase inhibitors has been shown to improve endothelium-dependent relaxation in the coronary arteries of patients with atherosclerosis [57]. On the other hand, improved blood flow by changes in blood rheology induced by LDL-apheresis may also contribute to the improvement of coronary flow [58]. However, reductions of fibrinogen and most other coagulation factors last not longer than 24 to 48 hours [59], whereas changes in blood viscosity have been measured until 1 week after an apheresis using dextran sulfate adsorption [60]. Since the follow-up assessments at the end of the study in LAARS were done 3-4 weeks after the last LDL-apheresis, rheologic changes did not confound our results. This indicates that functional improvements of the coronary vasomotor function on a level beyond the resolution of the angiogram may precede anatomic changes in severely stenotic coronary arteries [27,61,62]. Therefore, functional measures should be considered as additional, and possibly more sensitive outcome variables for short-term angiographic studies. In our study we also assessed the videodensitometric measurements of the blood flow in the coronary microcirculation as functional primary outcome variable. These data are presently being analyzed and will be published separately.

It has been shown that culprit lesions in unstable angina have increased vasoreactivity, responsible for the risk of recurrence of unstable angina or infarction [63]. Plaque stabilization by lipoprotein manipulation may require more than 1 year of aggressive treatment before a significant reduction in clinical events can be documented [15]. In our study we were confronted with a few myocardial infarctions in the apheresis group in the early phase of the study, a difference with the medication group which may be associated with the use of anticoagulants or platelet aggregation inhibitors. It must be emphasized that our study was not designed to evaluate the clinical events. However, combining both episodes of unstable angina and myocardial infarctions no differences between both treatment groups were observed. Considering the natural progression of CAD, it is notable that both treatment groups showed less events than expected in the second year of treatment. This observation supports the notion that the effect of cholesterol lowering on functional improvement precedes anatomic regression of atherosclerosis [27].

Conclusions

Combined LDL-apheresis and cholesterol lowering drugs in patients at high risk for cardiovascular events arrests further progression of CAD and induces functional improvement of the coronary blood flow. Studies of longer intervention periods are warranted to confirm these findings and observe the expected angiographic regression of CAD. However, only primary hypercholesterolemic patients with established CAD refractory to drug treatment should be treated with LDL-apheresis.

Acknowledgments

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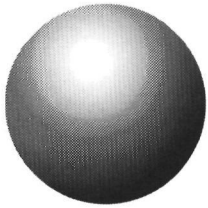

Chapter

Seven

Low density lipoprotein apheresis improves regional myocardial blood flow in patients with hypercholesterolemia and extensive coronary artery disease. The LDL-Apheresis Atherosclerosis Regression Study (LAARS)

Provisionally accepted for publication as:

Low density lipoprotein apheresis improves regional myocardial blood flow in patients with hypercholesterolemia and extensive coronary artery disease. The LDL-Apheresis Atherosclerosis Regression Study (LAARS)

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J Am Coll Cardiol 1996

Background: In patients with severe hypercholesterolaemia diet and lipid-lowering drugs are often insufficient to achieve optimal low density lipoprotein (LDL) cholesterol values. LDL-apheresis is a very effective lipid lowering therapy. Assessment of regional myocardial blood flow enables the evaluation of the functional state of the coronary circulation.
Methods: We studied 42 patients with severe hypercholesterolemia and extensive coronary artery disease who were randomized to diet and simvastatin with or without biweekly LDL-apheresis. Regional myocardial blood flow was assessed by digital subtraction angiography (DSA) with videodensitometric calculation of hyperemic mean transit time of contrast (HMTT), at baseline and after 2 years of therapy.
Results: LDL-cholesterol decreased by 63% (to 3.0 $mmol.l^{-1}$) in the LDL-apheresis (L) group and by 47% (to 4.1 $mmol.l^{-1}$) in the medication (M) group. Paired HMTT measurements were assessed in 43 regions in the L group and 35 regions in the M group. Baseline HMTT values of both groups were not significantly different. In the L group the mean HMTT of all regions decreased over 2 years from 3.35±1.18 to 2.87±0.82 s, -14% (p= 0.001), whereas no change in the M group was observed, 2.95±1.06 to 2.96±0.90 s (NS). In the patient-based comparison the mean change in HMTT was -0.45 s, -14% (p= 0.01) in the L group and -0.05 s, -2% (NS) in the M group, respectively.
Conclusions: Biweekly LDL-apheresis plus simvastatin decreased time-averaged LDL-cholesterol levels by an additional 31% (1.1 $mmol.l^{-1}$) in comparison with medication alone. Regional myocardial blood flow improved in the LDL-apheresis group and remained unchanged in the medication group, which is in accordance with previously reported improvement in exercise testing. The increase in regional myocardial blood flow is probably explained by the improvement in endothelial dependent and/or independent relaxation. For evaluation of lipid lowering therapy, functional assessment of the coronary circulation by DSA with videodensitometric calculation of the HMTT may be a more sensitive measure than quantitative coronary angiography, because it evaluates the overall effect on myocardial perfusion.

Introduction

Low density lipoprotein (LDL) cholesterol levels often remain elevated in patients with familial hypercholesterolemia (FH) despite diet and cholesterol lowering drugs. High LDL-cholesterol levels are strongly associated with premature development of coronary artery disease especially in males with inherent risk of coronary death [1]. LDL-apheresis is considered a valuable therapeutic option for patients with homozygous FH and for patients with coronary artery disease and hypercholesterolemia refractory to diet and drugs [2-4]. Plasma exchange or LDL-apheresis showed in a number of non-randomized studies regression or slowing of progression of coronary atherosclerotic lesions and resolution of xanthomas [2,5-8]. The use of plasma exchange was also associated with increased survival among homozygous FH patients in comparison with their untreated homozygous siblings [9]. In one recently published randomized trial in patients with FH, biweekly LDL-apheresis versus drug therapy during two years showed no difference in coronary anatomy as measured by quantitative coronary angiography (QCA) [10]. In the LDL-Apheresis Atherosclerosis Regression Study (LAARS), we neither found differences in quantitatively assessed coronary anatomy, however patients on LDL-apheresis had a significant improvement in exercise parameters versus patients on drug therapy alone [11]. Questions remained upon the cause of this functional improvement and the methods to evaluate the potential benefits in aggressive lipid lowering therapy. In lipid lowering trials, hard clinical endpoints such as improved survival and reduction of major cardiac events are generally accepted outcome measures, however, they require numerous patients over a long intervention period [12-15]. At the moment, QCA is the best surrogate endpoint for evaluation of lipid lowering therapy in coronary artery disease [16-22]. The application of QCA, however, has several limitations such as the diffuse character of coronary artery disease, the complexity of stenosis geometry, the foreshortening in non-orthogonal views, the insufficient contrast staining and the limited resolution of the radiographic chain [23-26]. Moreover, there seems to be a dissociation between the results of angiographic studies and clinical outcome. Lipid lowering therapy has shown to reduce the incidence of cardiovascular events without marked changes in coronary anatomy [22]. Treatment of hypercholesterolemia has shown to stabilize minor atheromatous plaques by depleting the lipid core. Therefore, not the change in angiographic lumen size, but plaque stabilization appears to be related to the reduction of clinical events [27].

Coronary atherosclerosis and hypercholesterolemia are associated with dysfunction of endothelium mediated vasomotion which impairs coronary and myocardial blood flow [28-32]. Lipid lowering therapy improves the endothelium mediated vasomotion after relative short periods of time [33-35]. The functional improvement of the coronary circulation has been shown to precede structural improvement [36]. The cumulative effects of all anatomical and functional abnormalities of the epicardial coronary arteries

in combination with possible dysfunction of conduit and resistance vessels are responsible for the ultimate effect of atherosclerosis on myocardial blood flow. In order to evaluate the functional state of the coronary circulation after aggressive lipid lowering with LDL-apheresis, we assessed regional myocardial blood flow by means of digital subtraction angiography (DSA) followed by videodensitometric calculation of the hyperemic mean transit time (HMTT) of contrast in a randomized study.

Patients and Methods

Subjects and treatment

The purpose of this study was to compare the effect of aggressive lipid lowering treatment by LDL-apheresis versus conventional treament on the anatomy and functional state of the coronary tree. The trial design, patients characteristics, results of QCA and exercise testing have been described previously [11]. Briefly, consenting patients with extensive coronary artery disease and hypercholesterolemia were randomized to diet and 40 mg simvastatin once daily with or without biweekly LDL apheresis. The inclusion criteria were 1. male patients between 18 and 70 years, 2. family history of hypercholesterolemia. 3. extensive coronary atherosclerosis demonstrated with heart catheterization. 4. serum total cholesterol of $\geq$8.0 mmol.l^{-1} or LDL-cholesterol $\geq$5.8 mmol.l^{-1} and fasting serum triglycerides $\leq$5.0 mmol.l^{-1} during a standard lipid lowering diet. The study population consisted of 42 male patients with a mean age of 52.0±9.2 years. The elevated lipid levels were due to heterozygous FH in 32 patients (76%), 16 in each group. No homozygous FH patients were included in this study. In the LDL-apheresis (L) group 17 patients had 3-vessel disease and 4 patients 2-vessel disease, whereas in the medication (M) group 19 patients had 3-vessel disease and 2 patients 2-vessel disease (a vessel was considered as diseased when there was a $\geq$50% diameter stenosis in a major branch). Before entry into the study all patients had a two months run-in period. Anti-anginal medication was continued but lipid lowering medication was stopped. If total cholesterol remained elevated above the lower limit for inclusion into the study, heart catheterization and exercise testing were carried out. After heart catheterization patients were randomized in a stratified way, which took into account the total cholesterol (TC) level, Lipoprotein(a) [Lp(a)] level, age and the history of coronary bypass surgery. Antianginal medication was continued at the same doses during the trial. If adaptation in medication was necessary during the study, the original prescription, if possible, was restored before the follow-up heart catheterization. The follow-up heart catheterization was performed one month after the last LDL-apheresis. Hemoglobin and hematocrit were measured just before the heart catheterization. LDL-apheresis was performed with an automated system MA-01 (Kanegafuchi Chemical Industry Co. Ltd, Osaka, Japan) with two small-sized dextran sulfate cellulose columns that were used and regenerated alternately in one procedure, permitting continuous

apheresis [11]. The ethical committee of the University Hospital Nijmegen approved the study.

Assessment of regional myocardial blood flow

To compare the maximal regional myocardial perfusion DSA of the coronary tree was followed by videodensitometry and calculation of the HMTT of contrast. Regional perfusion was assessed in the areas of the left anterior descending artery (LAD), circumflex artery (RCX) and right coronary artery (RCA) at baseline and at follow-up. This method has been developed in our laboratory and has been validated in animal model and human studies [37-41]. Before heart catheterization patients were trained to hold their breath, with use of a nose clamp at maximal inspiration during 15-20 seconds. After the completion of the left coronary angiography the left Judkins catheter with 7F tip (Cordis) was left in place for the DSA protocol in 60° LAO position. After angiography of the right coronary artery the protocol was repeated in 30° RAO position. Meticulous care was taken for the follow-up DSA to repeat it under exactly the same circumstances, with the same medication.

At the start of the procedure a 5F stimulation catheter was positioned in the right atrium. During DSA the heart was triggered in synchrony with the radiographic pulses, slightly above its inherent rate, to provide a strictly regular heart rhythm. Iohexol-350 (Nycomed AS, Oslo, Norway), a non-ionic low osmolar contrast agent was used. For all studies, 6 ml of contrast was injected, using a power injector at a speed of 4 $ml.s^{-1}$. Twenty five seconds after administration of 8-12 mg papaverine (depending on the size of the vascular bed) the patient was instructed to hold his breath, and image acquisition was started, 5 seconds later followed by contrast injection. One image per heart cycle was obtained just before the onset of the QRS complex. Image acquisition was performed on a Bicor radiograph system connected to a Digitron-3 computer for digital subtraction angiography (Siemens AG, Erlangen, Germany). Images were generated with the automatic brightness control switched off after the fourth image in every study to enable density calculation. Subsequently, the images were digitized in a 512 x 512 matrix with 1,024 density levels. The image quality was checked immediately after image acquisition and if necessary the imaging protocol was repeated with a maximum of 3 times.

Image and data processing were performed off line using a Sun SPARC station 10 (Sun Microsystems Inc. Mountain View, USA) with programmed Khoros software. Circular regions of interest (ROI) of 200-600 pixels were constructed over the tip of the coronary catheter to record the contrast injection and over the myocardium supplied by the respective vessels (Fig. 1). The ROIs were chosen at the myocardial level on predefined localizations, carefully avoiding overlap with major sidebranches and veins. Close to the myocardial ROI a background ROI was chosen to analyze changes in background density (Fig. 1). Time-density curves were generated by sampling the

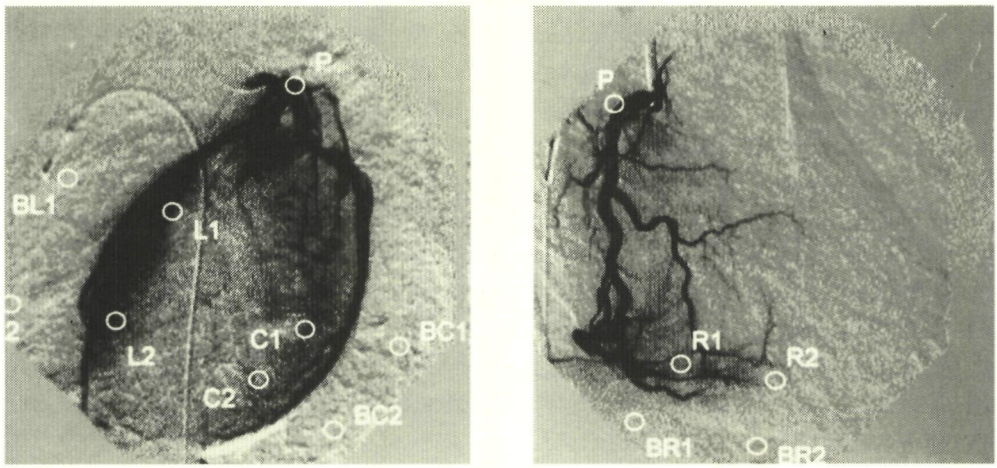

Fig 1. Example of the positioning of the ROIs in one of the subtracted images of the coronary arteries, early after contrast injection. In the left panel the left coronary artery in the 60° LAO projection and in the right panel the right coronary artery in the 30° RAO projection is shown

averaged pixel density within a ROI in consecutive images. Subsequently, the time-density curves were fitted with a gamma-variate function from the onset of the curve to the instant at which the descending part of the curve became less than 40% of its peak value [42]. In case of deviating background density, the fitted curves of the myocardial ROIs were corrected with the corresponding background ROI. The HMTT is the difference of the mean transit time of the myocardial ROI and the ROI at the injection site (P ROI). Because of the pressure dependency of flow during maximal hyperemia, HMTTs were corrected for a normalized mean aortic blood pressure of 100 mmHg for comparison between the two studies. This correction was performed by multiplying the mean transit time in seconds by the ratio $P\bar{a}/100$, where $P\bar{a}$ is the actual mean blood pressure during image acquisition. If a pressure curve showed a pressure drop or wedging during hyperemia, indicating that the catheter influenced maximal flow, the study was excluded from further analysis.

Time-density curves of particular ROIs or entire videodensitometric studies of insufficient quality were skipped for final analysis. During the trial all DSAs were evaluated and ROIs were chosen. Time-density curves were made but mean transit times were not calculated. At follow-up videodensitometry was repeated at identical conditions and analysis was performed in accordance with the original ROI overlay. Figure 2 shows an example of paired time-density curves of a ROI. For final analysis paired time density curves of sufficient quality without artifacts were selected by one

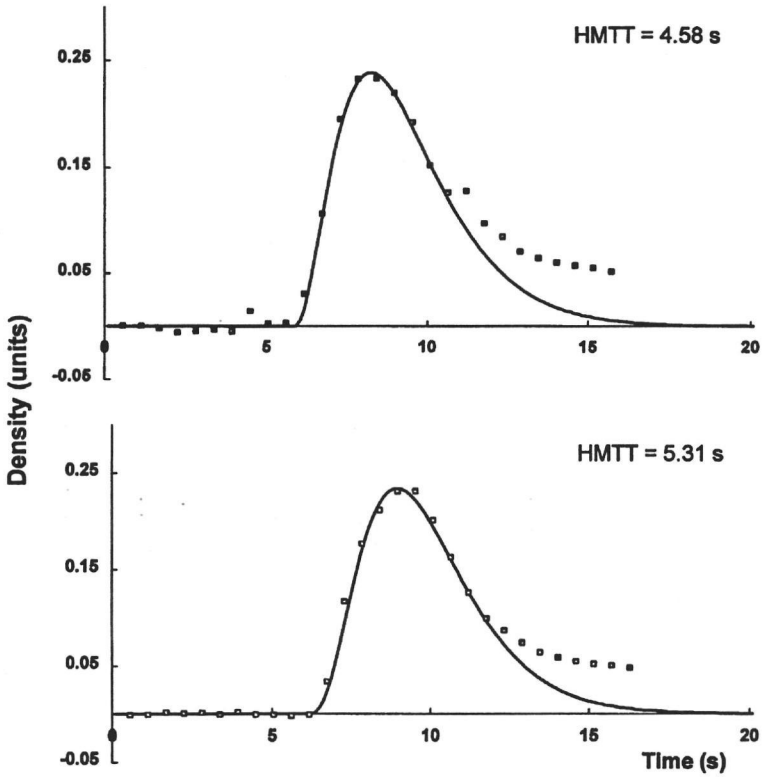

Fig 2. Example of paired time-density curves of a ROI at baseline (upper panel) and at follow-up (lower panel). The HMTT values are calculated from these curves, where the onset of the time is derived from the time-density curve of the respective ROI at the injection side

cardiologist (W.A.) blinded to treatment allocation. The final database was composed and subsequent HMTTs were calculated and corrected for blood pressure. Regional myocardial perfusion was assessed by averaging the comparative HMTTs (1-2 ROIs) in the area supplied by the left descending artery (LAD), the circumflex artery (RCX) and the right coronary artery (RCA) to one value per perfusion area per session. All perfusion areas including areas supplied by bypass grafts were imaged by the DSA protocol. ROIs of regions supplied by bypasses were classified to the original coronary artery region. Effect analysis of both lipid lowering treatments was a region-based assessment and a patient-based assessment of the change in myocardial perfusion. The patient-based evaluation was performed by averaging the regional HMTT values to one value per patient. Analysis of the patient-based HMTT was performed both with and without correction for change in hematocrit. For this correction the HMTT values at follow-up were subtracted by the % change of the hematocrit, assuming a linear relation between hematocrit and blood viscosity in this narrow range of actual hematocrit values [43].

Statistical analysis

Comparisons of pre-specified continuous variables within the groups were done by two-sided paired *t*-tests, between group analysis by unpaired *t*-tests for normally distributed data. For categorical comparison a chi^2 test was done when appropriate. Correlations were tested by assessing the Pearson correlation coefficient. For all hypotheses tests, a two-sided *p*-value of less than 0.05 was considered significant. Results are expressed as means ± SD, unless otherwise indicated.

Results

From March, 1990 to May, 1992, 42 male patients were randomized into two groups of 21 patients in our center. The follow-up was completed in June 1994. The results of both groups on lipid metabolism, QCA and exercise testing have been described previously [11]. In short, LDL-cholesterol decreased in the L group from 7.78±1.86 to a time-averaged value of 2.95±1.13 $mmol.l^{-1}$ and in the M group from 7.85±2.34 to 4.13±1.58 $mmol.l^{-1}$. The mean change in lipid values during the study are depicted in Figure 3. QCA showed no changes in mean segment diameter (MSD) and minimal

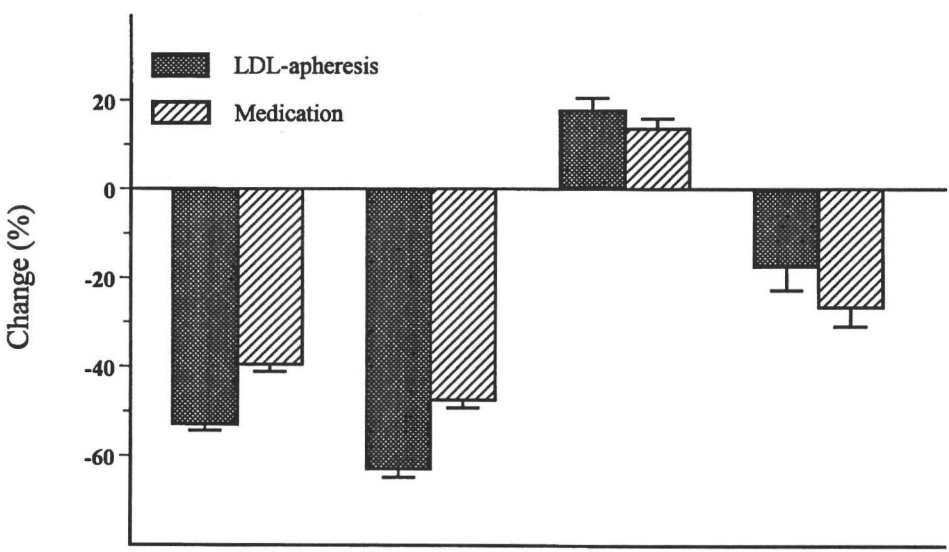

Fig 3. Bar graphs showing the change from baseline to the mean value during the study of total cholesterol (Tot. chol.), low density lipoprotein cholesterol (LDL-chol.), high density lipoprotein cholesterol (HDL-chol.) and triglycerides. The values are expressed as mean percentage change ± SEM. In the LDL-apheresis group Tot. chol. and LDL-chol. are presented as time-averaged values

Table 1 Clinical characteristics and patient based-study data

pat* (nr)	group L/M†	age (yr)	MI / CABG PTCA‡	LDL-chol (mmol l⁻¹) base	LDL-chol (mmol l⁻¹) during§	change MSD (mm)	change MOD (mm)	change ST-time (s)	change HMTT (s)
1	L	29	-	12 09	5 67	0 39	0 20	-420	0 17
2	M	59	I/C	7 01	3 74	0 14	0 15		-0 09
3	M	67	A/C	6 62	3 90	0 11	0 05	0	0 97
4	M	59	I/	7 05	4 26	0 06	0 04		1 46
5	M	52	I/	5 94	3 58	-0 14	-0 12		-1 58
6	L	43	NQ/	5 45	2 65	-0 30	-0 24	-30	-0 19
7	M	69	I/CP	6 28	3 06	0 11	-0 09		
8	M	67	I/P	6 51	2 69	-0 42	-0 25		2 57
9	L	46	-	6 63	3 00	-0 12	-0 09	-360	-0 62
10	L	65	-	6 97	3 76	0 05	0 07		-0 14
11	L	46	I/C	7 17	2 76	0 23	0 19		
12	M	46	A/	6 11	3 57	-0 08	-0 08	60	-0 75
13	L	66	I/	6 97	2 14	-0 08	-0 16	-299	
14	M	58	/C	6 43	3 47	0 07	0 12		
15	L	53	I/C	6 80	1 98	-0 09	-0 14	-360	-0 38
16	M	40	-	9 90	5 32	0 26	0 20	120	0 00
17	L	58	I/C	6 71	2 03	0 00	-0 13	-60	-0 51
18	L	52	A/C	11 36	5 22	-0 33	-0 06	23	-0 06
19	M	47	I/C	12 86	5 62	-0 29	-0 09		-0 41
20	L	30	A/C	10 75	3 69	-0 03	-0 06	-330	0 41
21	L	50	A/C	7 60	3 12	0 04	0 20		
22	M	45	NQ/	9 20	4 95	0 07	0 10	0	-0 21
23	M	56	A/C	11 65	4 93	0 17	0 07		-0 62
24	M	44	I/	6 59	3 43	0 18	0 06	360	-0 14
25	L	57	I/P	7 06	2 09	-0 02	-0 06	-165	-1 09
26	M	56	I/C	5 92	2 76	0 08	0 05	-120	-1 58
27	L	50	I/	10 84	3 36	-0 06	-0 04		-0 30
28	L	42	A/	7 60	2 51	0 20	0 17		0 45
¶29	L	63	I,A/C	6 57	2 09				-1 27
30	M	49	/CP	8 30	4 29	0 00	0 03		-1 50
31	M	52	I/	7 60	4 14	-0 01	0 02	60	0 22
32	L	54	I/C	6 28	2 10	0 10	0 09	-240	-1 51
33	L	42	-	7 03	2 00	-0 14	-0 11		-0 50
34	M	56	A,A/C	6 68	3 26	-0 06	-0 08	0	0 59
35	L	56	A/C	6 66	2 03	-0 11	-0 13	-55	-1 34
36	L	46	-	8 84	4 02	0 00	0 03	-67	-0 36
37	M	49	NQ/	7 31	4 48	0 07	-0 01		
38	M	50	A/CP	5 83	2 79				0 24
39	L	62	I/PC	6 36	2 59	-0 03	0 03	-60	-1 37
40	L	54	I/	6 38	2 27	0 03	-0 01	-88	0 57
41	M	66	A/	7 16	3 16	-0 02	-0 07	-60	
42	M	42	I/P	13 90	9 29	0 20	0 15	5	-0 03

**pat, patient, † L, LDL-apheresis group, M, medication group, ‡,all events before entrance in the study, MI, myocardial infarction, localization of myocardial infarction, A anterior, I inferior, NQ non-Q wave myocardial infarction. CABG, coronary artery bypass surgery, "C" PTCA, percutaneous transluminal coronary angioplasty, abbreviation "P" LDL-chol, Low density lipoprotein cholesterol §, mean time averaged LDL-cholesterol level (for conversion of cholesterol values to mg/dl, multiply by 38 7) Change, difference in value between follow-up and baseline, MSD, mean segment diameter from QCA measurements, MOD, minimal obstruction diameter, ST-time, time to 1 mm (0,1mV) ST-segment depression, HMTT, hyperemic mean transit time ¶ The patient who had a re-CABG 12 weeks after the start of LDL-apheresis and died post-operative due to low cardiac output*

Table 2. Regional HMTT values in the 3 myocardial perfusion areas

	LAD region	RCX region	RCA region	All regions
LDL-apheresis group	n = 17	n = 18	n = 7	n = 42
baseline HMTT (s)	3.28 (0.97)	2.93 (0.66)	4.61 (1.85)	3.35 (1.18)
follow up HMTT (s)	2.81 (0.86)	2.66 (0.67)	3.57 (0.79)	2.87 (0.82)
p-value	0.02	0.08	0.11	0.001
Medication group	n = 15	n =13	n = 7	n = 35
baseline HMTT (s)	2.78 (0.78)	3.15 (1.22)	2.95 (1.35)	2.95 (1.06)
follow up HMTT (s)	2.75 (0.81)	3.11 (1.07)	3.14 (0.79)	2.96 (0.90)
p-value	0.88	0.93	0.74	0.96
*Difference (p-value)**	0.17	0.51	0.14	0.04

*Data represent means; standard deviation between brackets; HMTT, hyperemic mean transit time. *difference in mean change between LDL-apheresis and medication group (t-test)*

obstruction diameter (MOD) within and between the two groups. At exercise tests in the L group versus the M group, time to the onset of 1 mm (0.1 mV) ST-segment depression was prolonged by 39% and maximal ST-segment depression was halved. The clinical characteristics and patient-based study data are listed in Table 1.

Hyperemic mean transit time (HMTT)

The DSA protocol for calculation of the HMTT was performed in all 42 patients. In the L group 19 patients completed the 2 years of LDL-apheresis, and 2 patients had to stop because of progression of angina pectoris after 3 and 10 months, respectively. Both patients underwent coronary artery bypass surgery (CABG). In the M group one patient underwent CABG after 24 months of treatment. The HMTT studies of these patients at follow-up were considered as end of study measurements. One patient in the L group who was hospitalized for unstable angina pectoris, 12 months after the start of the LDL-apheresis, underwent percutaneous transluminal coronary angioplasty (PTCA) of the LAD and the RCA. After PTCA LDL-apheresis was continued, but the two perfusion areas were excluded for assessment of regional myocardial perfusion. In 35 patients (83%, 18 in the L group and 17 in the M group) comparative data of the first study and subsequent follow-up were available. The reasons for missing of paired data in 7 patients were as follows: 1. insufficient image quality of one session in 2 patients and of both sessions in a third patient with chronic airway obstruction, 2. loss of stored digital images of one session in 2 patients, and 3. no comparable DSA in 2 patients due to abusive recordings. In total 121 comparative ROIs were available, 68 in the L group

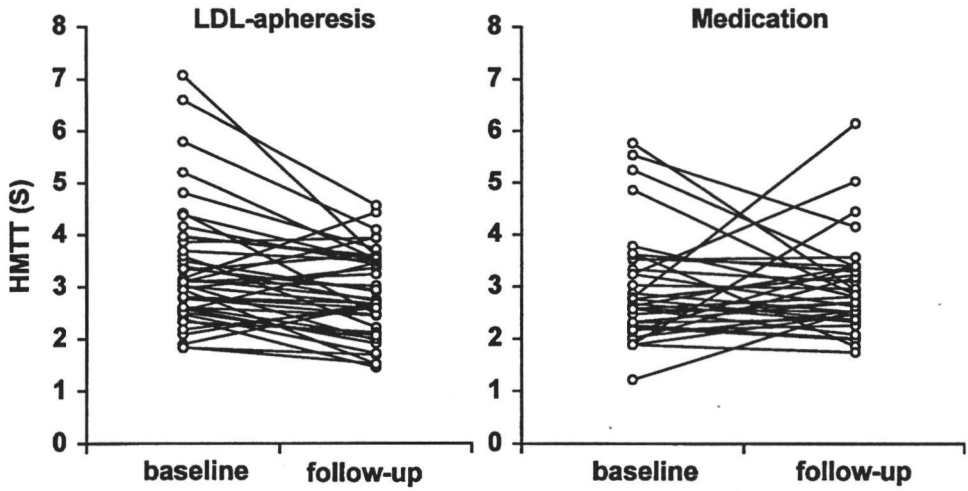

Fig 4. Plots showing the changes of regional HMTT in the LDL-apheresis group (n=42) and medication group (n=35)

and 53 in the M group, respectively, mean 3.5±1.7 per patient. One-hundred three ROIs were generated in regions supplied by native coronary arteries and 18 supplied by bypasses. Based on ROIs, the average HMTT decreased by 0.59±1.01 s in the L group ($p<0.001$) and decreased by 0.10±1.16 s ($p=0.52$) in the M group; the difference between the two groups is 0.48 s ($p= 0.01$).

For analysis of the regional myocardial blood flow, ROIs (1 to 2) belonging to the same region were averaged. In Table 2, the results of the HMTT, based on regions are listed. The baseline HMTT values of the L group and M group were 3.35±1.18 s (n=42) and 2.95±1.06 s (n=35), respectively, and were statistically not different ($p=0.52$). Changes of all regional HMTTs in both groups are depicted in Figure 4. The relative changes in HMTT of the three perfusion areas of the LAD, RCX and RCA, respectively, are shown in Figure 5. The distribution of regions in the L group was; LAD: 17, RCX: 18 and RCA: 7 regions and for the M group 15, 13 and 7, respectively. All three coronary perfusion areas contributed to the same extent to the results.

In the patient based analysis the averaged HMTT value in the L group (n=18) was 3.19±0.78 s at baseline and 2.74±0.68 s at follow-up, mean difference -0.45 s, -14% ($p=0.01$). In the M group (n=17) the averaged HMTT value was 2.99±0.84 s at baseline and 2.94±0.77 s at follow-up, mean difference -0.05 s, -2% ($p=0.85$). The mean difference in change between the two groups was 0.40 s ($p=0.19$). Hematocrit measured at heart catheterization was in the L group 0.44±0.03 at baseline and 0.43±0.04 $l.l^{-1}$ at follow-up ($p=0.23$) and in the M group 0.45±0.03 and 0.45±0.03 $l.l^{-1}$, respectively

(p=0.44). For correction of a possible change in blood viscosity due to a change in the hematocrit, the mean patient based HMTT at follow-up catheterization was corrected by the percentage change in hematocrit from baseline to follow-up catheterization. In the L group (n=18) the averaged HMTT corrected for hematocrit change decreased from 3.19±0.78 to 2.78±0.69 s (p=0.008) and in the M group from 2.99±0.84 to 2.97±0.84 s (p=0.94) The mean difference in HMTT between the L group and the M group after correction for hematocrit was 0.38 s (p=0.22).

There were no significant correlations between the change in HMTT with the mean lipid levels during the trial [total cholesterol, LDL-cholesterol, high density lipoprotein (HDL) cholesterol, ratio LDL/HDL, Lp(a)] or change in lipid levels. No significant correlations were found between change in HMTT and change in MOD, MSD or change in time to 1 mm ST-segment depression. Categorizing patients into groups with improvement versus impairment of time to 1 mm ST-segment depression at the exercise test showed a decrease in HMTT of 16±21% in the group with improvement, versus an increase in HMTT of 4±19% in the group with impairment (p=0.04). The change in hematocrit at heart catheterization showed no relation with the time to 1 mm ST-segment depression nor with the change in HMTT.

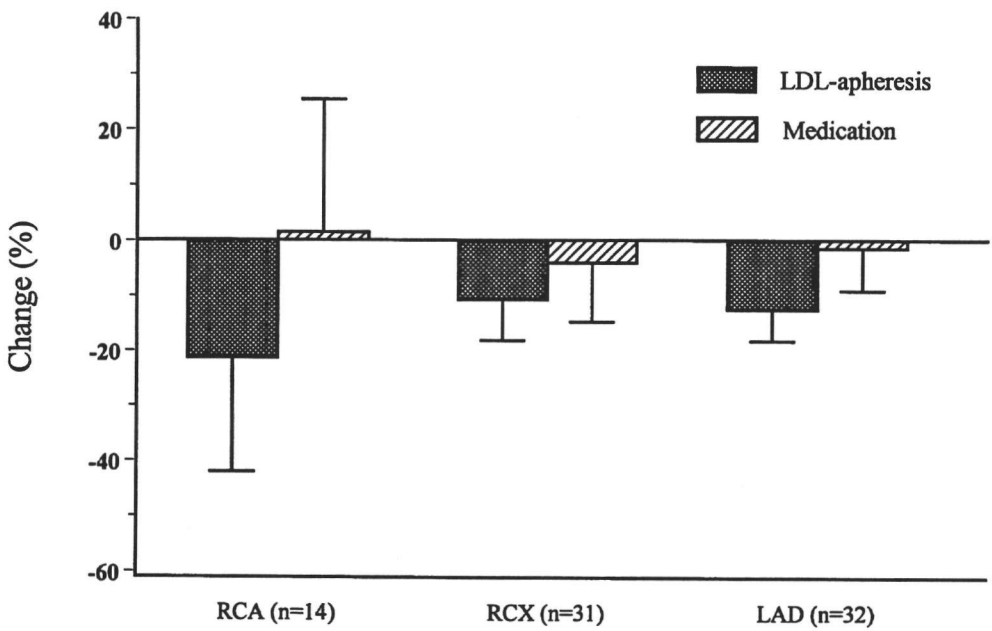

Fig 5. Bar graphs showing the mean percentage change of HMTT ± SEM in the regions of the left anterior descending artery (LAD), the circumflex artery (RCX) and the right coronary artery (RCA), respectively, for the LDL-apheresis group versus the medication group

Discussion

Although LAARS showed no significant change in quantitatively assessed coronary anatomy in both groups, the results of the HMTT measurements showed an increase in myocardial perfusion in the group of patients treated with LDL-apheresis, whereas patients on medication only remained stable. This improvement in myocardial perfusion in the group on LDL-apheresis is in agreement with the improved ischemic threshold as has been published before [11,44]. These results show a discrepancy between anatomical and physiologic parameters in the assessment of coronary atherosclerosis. The improved functional status in the patients on LDL-apheresis is accompanied by a lower LDL-cholesterol level (1.1 $mmol.l^{-1}$) than in the group of patients on medication only. To explain the improvement in myocardial perfusion we hypothesized that one or more of the following mechanisms may be operative:

1. Recovery of endothelial NO production and release in the conduit vessels. Recent studies in animals and humans associate hypercholesterolemia and increased Lp(a) levels with an impaired endothelium-dependent vasodilatation [28-32,45]. Lipid lowering during 6 months to one year may enhance the endothelial function as tested with acetylcholine [33-35]. Improvement in endothelial function may enhance exercise induced dilatation of the large epicardial coronary arteries [28,46]. The more pronounced lipid lowering in the group with LDL-apheresis may result in a better recovery of endothelial function and may thus improve the ischemic threshold at exercise testing. Assessment of HMTT reveals a time parameter that is inversely related to maximal myocardial perfusion. Due to the method of assessment of the HMTT (intra-coronary papaverine and sublingual isosorbide dinitrate), it is unlikely that endothelium dependent vasodilatation is involved in the improved regional myocardial blood flow in this study.

2. Remodeling of the conduit vessel wall. Regression of the atherosclerotic plaques initially leads to remodeling of the vessel wall, without a significant effect on plaque size and/or cross-sectional area of the lumen of the epicardial coronary arteries [27,47,48]. The functional response of the remodeled coronary arteries to dilate in reaction to papaverine, however, may be enhanced and maximal myocardial perfusion may be increased. This view is supported by a recent publication in which the epicardial response to intra-coronary nitroglycerin administration decreased significantly with increasing atherosclerotic wall thickening [49]. Next to obstruction of the vascular lumen atherosclerosis of the arterial wall also has an endothelium independent effect on coronary vasomotion and flow. This may explain the observation that functional improvement precedes anatomical regression of atherosclerosis.

3. Improved arteriolar vasodilatation. Resistance vessels are spared from the development of overt atherosclerosis. Relaxation of these vessels, however, may be reduced in patients with high LDL-cholesterol levels due to alterations in endothelial

cell function as established in primates [30]. Endothelium dependent arteriolar relaxation was impaired, whereas endothelium-independent responses were nearly identical [30]. In the human forearm model the impairment of the microvascular vasodilator function in patients with hypercholesterolemia is only endothelium dependent [32]. So improvement in endothelium independent arteriolar vasodilatation is highly speculative. Another hypothesis introduced by Gould et al [50] was that the coronary flow response induced by a direct effect of an arteriolar vasodilator may be augmented by a further improved flow-mediated, endothelium dependent epicardial coronary artery vasodilatation. This effect, however, was not likely to occur in this study where patients received prior to angiography isosorbide dinitrate for epicardial coronary artery vasodilation. Maximal epicardial vasodilatation after isosorbide dinitrate sublingually is known to persist for 30 to 60 minutes which was the time we needed to perform the angiography and DSA [51,52].

4. Changes in blood rheology. Improved myocardial perfusion may also be due to changes in blood rheology as a result of repeated LDL-apheresis [53,54]. Because the assessment of HMTT took place one month after the last LDL-apheresis, a direct effect of LDL-apheresis can be neglected. During the study there was a small decrease in hemoglobin level in the group of patients on LDL-apheresis but at heart catheterization the hematocrit levels were not significantly different in comparison to baseline. The results of the HMTT analysis after correction for change in hematocrit were virtually unchanged, so we decided to use the original data set for the entire analysis. During LDL-apheresis using dextran sulfate cellulose columns plasma fibrinogen, another determinant of viscosity, is lowered acutely by 26 - 35%, returning to pretreatment levels between 2 and 7 days [11,55]. Rigidity of red blood cells is increased in patients with high LDL-cholesterol values causing an increase in blood viscosity. After analysis of the HMTT with correction for the hematocrit and the delay between the last LDL-apheresis and the follow-up DSA, we conclude that there was no significant effect of LDL-apheresis on blood viscosity at the moment of the assessment of the HMTT.

The large individual relative change in HMTT compared with the relative change in MSD derived from QCA can be explained by the fact that the mean transit time is inversely related to the 4th power of the radius of a coronary vessel. In a model with a constant pressure and non-pulsatile flow, a 12% increase in coronary diameter in a 3.0 mm coronary artery equals an 58% increase in coronary flow. Reproducibility of the HMTT under identical circumstances in the past has been proved to be good [40].

The improvement in HMTT was in agreement with the results of exercise testing, although there was no close correlation between the individual measurements. The different methodology of testing and differential effects of exercise and papaverine induced vasodilation may explain the individual differences in the response to LDL-apheresis. There was also no close relation between change in coronary anatomy and exercise parameters. This correlation is known to be highly variable [56]. The functio-

nal impact of lipid lowering interventions can be evaluated by other techniques such as intracoronary Doppler and nuclear tests such as thallium-201 single photon-emission computed tomography (SPECT) and positron emission tomography (PET). PET has the potential to measure quantitatively myocardial blood flow and lipid lowering studies with PET evaluation are now underway [57]. Coronary vasomotion after acetylcholine can be assessed by QCA. Assessment of coronary or myocardial blood flow induced by different vaso-active stimuli, in combination with QCA and/or intra coronary echocardiography, may further elucidate the mechanisms that are underlying the clinical improvement in this study.

Limitations of the present study may be the following. The hyperemic mean transit time is inversely related to flow if the vascular volume between the tip of the catheter and the regarding region of interest is constant ($t = V/F$ where t = mean transit time, F = flow and V = vascular volume between the site of injection and point of measurement). Assessment of the HMTT was based on the assumption that the vascular volume after intra coronary papaverine was maximal and equal at the baseline and follow-up heart catheterization. Theoretically, if the vascular volume would be smaller due to atherosclerosis and the flow would remain the same, a shorter mean transit time could also be found that erroneously could be interpreted as favorable. This type of error is very unlikely because vascular volume is mainly determined by arterioles and capillaries, vessels which are not involved in the atherosclerotic process. In addition, the unchanged MSD in this study supports the assumption that the vascular volume of the conduit vessels remained unchanged.

In this study regional myocardial perfusion was assessed as the primary endpoint for flow evaluation. For the patient based HMTT, regions of one patient were averaged to one HMTT value. However, in some patients only one region was available, in one patient even based on only one ROI. In other patients 8 ROIs in 3 myocardial perfusion areas were available (including bypass evaluation). So the patient based HMTT value is not always representative for the entire myocardial perfusion in that patient.

The following conclusions can be drawn from this study. Biweekly LDL-apheresis plus simvastatin improved regional myocardial blood flow in patients with severe hypercholesterolemia and extensive coronary artery disease. In the control group treated with lipid lowering drugs only, regional myocardial blood flow remained stable. The improvement in myocardial blood flow is in agreement with the improvement in ischemic threshold at exercise testing. The additional decrease in LDL-cholesterol levels due to LDL-apheresis may be the explanation for the functional improvement of the coronary circulation, although minor rheologic changes can not be ruled out completely. The functional improvement may be due to endothelium dependent and/or independent changes in the coronary circulation. For the elucidation of the exact mechanism further studies are needed. The assessment of the regional myocardial blood flow by DSA and videodensitometric calculation of the HMTT is a feasible method for

the evaluation of lipid lowering trials. It requires some minor modifications of the radiographic equipment and instruction for breath holding for the patient. Assessment of the HMTT may be a more sensitive outcome measure than QCA for the evaluation of lipid lowering therapy because it evaluates the overall effect on myocardial perfusion.

Acknowledgments

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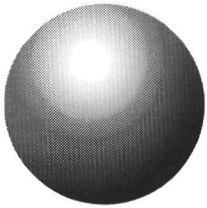

Chapter

Eight

The prevalence of peripheral vascular disease in familial hypercholesterolaemia

Published as:

The prevalence of peripheral vascular disease in familial hypercholesterolaemia

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Objectives. In patients with familial hypercholesterolaemia (FH) the prevalence of haemodynamically significant peripheral vascular disease (PVD) was measured in relation to lipoproteins, general risk factors, and the presence of coronary artery disease (CAD).
Design. A case control study.
Setting. The outpatient lipid clinic of a university hospital (tertiary referral centre).
Subjects. Patients with heterozygous FH [n=68; age 45.8±11.6 years; untreated LDL-cholesterol 9.2±2.0 mmol.L^{-1}] were compared with control subjects matched for gender, age, weight, smoking and presence of hypertension [n=27; age 44.0 years; LDL-cholesterol 3.8±1.3 mmol.L^{-1}].
Main outcome measures. PVD was assessed during cholesterol-lowering treatment using ankle/arm blood pressure ratios and analyses of Doppler-derived blood flow velocities in the femoral artery at rest and during reactive hyperaemia. The diagnosis CAD was assessed clinically.
Results. Haemodynamically significant PVD was found in 21 (31%) FH patients and in one (3.7%) control subject, predominantly localized in the femoro-popliteal vessels. CAD was present in 30 (44.1%) FH patients and in one (3.7%) control subject. PVD could be demonstrated in 50% of FH patients with CAD [relative risk 3.2 (95% CI 1.4 to 7.2)] and in 19% as the first manifestation of vascular disease. Males and females were equally affected. Mean arterial blood pressure of FH patients with PVD was higher compared to FH without PVD.
Conclusions. Haemodynamically significant PVD appears to be more prevalent in FH patients than is generally assumed, especially in those with CAD. A relation with lipoprotein levels could not be demonstrated.

Introduction

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder caused by one of several mutations in the gene on chromosome 19 coding for the low density lipoprotein (LDL) receptor [1,2]. The disorder is characterized by elevated levels of LDL cholesterol, tendocutaneous xanthomata and premature coronary artery disease (CAD) [1]. Individuals homozygous for the genetic defect have extremely high serum cholesterol levels and they mostly develop CAD and peripheral vascular disease (PVD) in the first or second decade of life [1,3]. Clinical manifestations of CAD in heterozygous subjects appear in over 50% prior to their fifties; men develop CAD in their third to fifth decade and women about 10 years later [4,5]. In contrast, PVD seems to occur only at a slightly increased frequency and is considerably less prevalent than premature CAD [4,6]. The prevalence of intermittent claudication in heterozygous FH has been reported between 8 and 16% [7-10]. Nevertheless, the occurrence of bruits over the femoral arteries have been described more frequently [8,10,11]. In a few studies, a decreased ankle/arm systolic pressure ratio has been found in 62-65% of asymptomatic heterozygous FH patients [10,11]. Apparently, PVD in heterozygous FH is much more prevalent subclinically, only causing symptomatology in advanced cases [12,13].

Sophisticated non-invasive ultrasonic Doppler techniques are now available to facilitate the diagnosis of PVD. The ankle/arm systolic pressure ratio at rest and during hyperaemia is a reproducible method to determine the presence of arterial insufficiency in the lower limbs [14]. To differentiate between haemodynamically significant stenotic segments located in the aorto-iliac or the femoro-tibial tract, measurement of ankle/arm systolic pressure ratio should be combined with the assessment of the haemodynamic status of the aorto-iliac tract. Usually, invasive intra-arterial pressure measurement in the common femoral artery in rest and during reactive hyperaemia are used to assess the functional status of the aorto-iliac tract (the "golden standard"). Several studies have shown that these invasive pressure measurements can be replaced by analysis of the blood flow velocities in the common femoral artery obtained non-invasively by Doppler spectrum measurements [12,15]. This method has proven to be an accurate, non-invasive, easy-to-perform screening-test to assess functionally and haemodynamically significant stenosis in the aorto-iliac tract. Moreover, the performance of Doppler spectrum analyses during reactive hyperaemia has been shown to enhance the sensitivity to detect haemodynamically significant aorto-iliac pathology [16].

The present study was undertaken to determine the prevalence of PVD by non-invasive measurements of the ankle/arm systolic pressure ratio and a Doppler spectrum analysis of blood flow velocities in the common femoral artery, both at rest and during reactive hyperaemia, in heterozygous FH patients and matched normocholesterolemic control subjects. The presence of PVD will be related to lipids and lipoproteins, general risk factors, and the presence of CAD.

Materials and methods

Subjects

A consecutive series of 72 unrelated patients known to have definite heterozygous FH were recruited from the outpatient lipid clinic of the St. Radboud University Hospital of Nijmegen, Nijmegen, the Netherlands, to which they had been referred by either general practitioners or hospital specialists. Four patients refused to take part in the study, one because of poor general health. The diagnosis familial hypercholesterolaemia was based on the following criteria: LDL cholesterol above ninetyfifth percentile for sex and age (in general >8.0 mmol.L^{-1}); the absence of a secondary hyperlipoproteinaemia; the presence of tendon xanthomata; signs or symptoms of CAD before the age of 55 in males or the age of 60 in females, and/or a first-degree family member with hypercholesterolaemia or tendon xanthomata or CAD before the age of 55 and 60 in males and females, respectively. The criteria for CAD were the presence of angina (history of exercise-associated chest pain), myocardial infarction (proven by electrocardiogram and/or serum enzyme changes), angiographically proven disease or a history of coronary artery bypass surgery. Hypertension was defined as systolic blood pressure >160 mmHg and diastolic blood pressure >90 mmHg in supine position at rest. Patients with diabetes mellitus were excluded (fasting glucose >6.0 mmol.L^{-1} and haemoglobin A1C >6.4%). The control subjects consisted of 27 volunteers from the same hospital, matched for gender, age, weight, presence of hypertension and smoking habits. They were selected on the basis of their serum total cholesterol levels (below 6.5 mmol.L^{-1}) and the absence of systemic or metabolic diseases.

Information was recorded from 68 FH patients and 27 control subjects. including the presence of angina and intermittent claudication, details of past medical history, family history, smoking status, alcohol consumption, previous treatment for hyperlipoproteinaemia, and present use of drugs. Medical records of the FH patients were used for verification of this information, including previous lipid and lipoprotein levels. Vital signs were measured, the body mass index was calculated, and the presence of tendon xanthomata, xanthelasmas, arcus cornealis and peripheral pulsus were noted.

Lipid and lipoprotein measurements

Fasting concentrations of total cholesterol, serum triglycerides, high-density lipoprotein (HDL) cholesterol, and LDL cholesterol were measured in both groups. For the FH group, mean levels of at least two measurements without lipid lowering treatment and four measurements during treatment with intervals of 2-3 months, were recorded before the vascular measurements were taken. Lipoprotein(a) [Lp(a)] levels were only measured during hypolipidemic drug treatment in the FH group. Serum total cholesterol and triglycerides were determined by enzymatic methods (CHOD-PAP, no. 237574,

Boehringer Mannheim GmbH, FRG and Sera-PAK, no. 6639, Miles, Milan, Italy, respectively). HDL-C was determined using the polyethylene glycol 6000 precipitation method [17]. LDL-C was calculated by subtraction. Lp(a) was measured by a specific radio-immunoassay (apolipoprotein(a) RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden).

Doppler spectrum analysis and ankle/arm systolic pressure ratio measurement

In one session, blood flow velocities in the common femoral arteries and ankle/arm systolic pressure ratios of both limbs were recorded, both at rest and during reactive hyperaemia, as described earlier [16]. In short, Doppler signals were obtained from the common femoral artery with an 8 MHz bidirectional continuous-wave Doppler apparatus (Medasonics Inc., Mountain View, CA, USA). The probe was placed just below the inguinal ligament. Reactive hyperaemia was induced by thigh cuff compression for 5 min at a pressure of at least 50 mmHg above the systolic arterial thigh pressure. Doppler spectra during reactive hyperaemia were obtained approximately 15 s after relief of the thigh compression. Doppler signals were processed by a real-time spectrum analyser (Radionics SA8000; Scarborough, Ontario, Canada) and subsequently digitally stored on a computer for off-line analysis. Maximum-frequency waveforms were calculated from the spectra and several parameters were calculated that describe the shape of the waveforms [18]. Based on a combination of six of these Doppler parameters [duration of the acceleration phase (T_{max}), slope of the deceleration phase (SL_{down}), maximum frequency of receding curve (F_{min}), and pulsatility index ($PI=F_{max}-F_{min}/F_{mean}$) of the at-rest spectra and duration of the acceleration phase (T_{max}) and resistance index ($RI=F_{max}-F_{dia}/F_{max}$) of the spectra during reactive hyperaemia], the presence of haemodynamically significant aorto-iliac pathology can be assessed accurately [16]. The same Doppler probe was used to determine the ankle/arm pressure index, using the systolic radial artery pressure and the highest pressure in either the dorsalis pedis or posterior tibial artery; the side with the highest radial artery systolic blood pressure at rest was used as reference side for pressures during reactive hyperaemia. Haemodynamically significant vascular disease was defined as an ankle/arm pressure index at rest <0.90 and/or a decrease of the pressure index during reactive hyperaemia ≥ 0.20.

Statistical analysis

Statistical analyses were performed with procedures available in the statistical package for social sciences (SPSS Inc., Chicago, IL, USA), using Student's *t*-test or the Mann-Whitney U-test for differences in means, and the Yates' corrected chi-squared test for differences in proportions. A *P*-value of less than 0.05 was considered to be significant. All results are expressed as mean±SD, unless indicated otherwise.

Results

Subjects

A total of 68 patients (29 males and 39 females) with heterozygous FH and 27 control subjects (13 males and 14 females) were examined. The age distribution of the FH population was comparable to the control subjects (Fig. 1). The mean ages were 45.8±11.6 years (range 22-68 years) for the FH patients and 44.0±10.9 years (range 24-64 years) for the control group. The main cardiovascular risk factors are summarized in Table 1. No significant differences were found between the FH patients and the control subjects for the body mass index, smoking habits, alcohol consumption and hypertension. The presence of CAD in the FH population was significantly higher than in the control group, 44.1% versus 3.7%, respectively ($P<0.001$) (Table 1). Twelve FH patients had a myocardial infarction in the past and 10 had undergone coronary artery bypass surgery. One subject in the control group had a myocardial infarction in the past and was free from angina pectoris. The cumulative frequency of CAD in FH patients as a function of age of onset is shown in Fig. 2 (a). The mean age of onset of CAD in the total FH group was 46.3±11.1 years (range 25-65 years, median 45 years), being 41.8±10.0 years and 53.2±9.1 years for men and women, respectively.

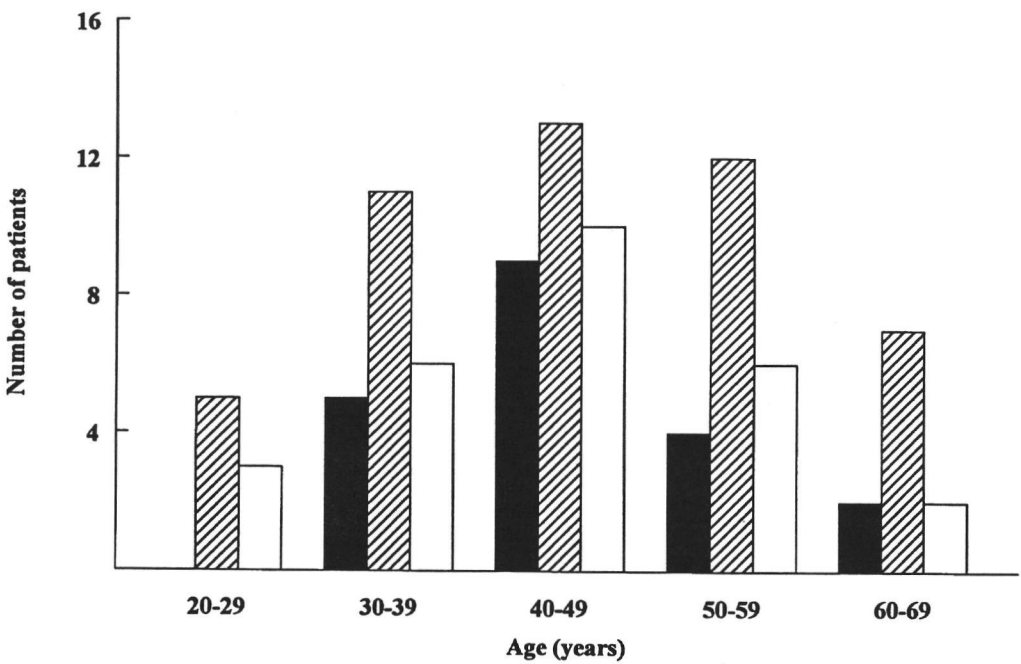

Fig. 1. Age distribution in different cohorts of patients with familial hypercholesterolaemia, with (filled bars) and without (hatched bars) peripheral vascular disease, and control subjects (open ba

Table 1. Characteristics of all patients with familial hypercholesterolaemia (FH_{TOTAL}), with (FH_{PVD+}) and without (FH_{PVD-}) peripheral vascular disease, and of control subjects[a]

Group	Sex (M/F)	Age (yrs)	BMI ($kg.m^{-2}$)	Smoking n (%)	Hypertension n (%)	MAP (mmHg)	Angina n (%)	ECG-changes n (%)	Claudication n (%)
FH_{TOTAL}	29/39	45.8±11.6	24.7±3.1	25 (36.7)	7 (10.3)	96±11	24 (35.3)*	30 (44.1)*	1 (1.5)
FH_{PVD+}	8/13	47.6±11.0	25.4±3.1	9 (42.8)	4 (19.0)	104±9§	11 (52.4)*	15 (71.4)#	1 (1.5)
FH_{PVD-}	21/26	45.8±10.8	24.3±3.0	16 (34.0)	3 (6.4)	93±11	13 (27.6)¶	15 (31.9)	0
Controls	13/14	44.0±10.9	23.7±2.4	12 (44.4)	2 (7.4)	97±9	0	1 (3.7)	0

*[a]Values are means±SD or numbers (percentages); M/F, male/female; BMI, body mass index; MAP, mean arterial pressure (normal range 80-100 mmHg); differences versus control subjects: ¶0.001≤P<0.01; *P<0.001; differences versus FH_{PVD-}: #0.001≤P<0.01; §P<0.001*

Lipids and lipoproteins

Without lipid lowering treatment, mean basal serum total cholesterol and LDL cholesterol levels in the FH group were strongly elevated (Table 2). HDL cholesterol concentrations were significantly lower and serum triglycerides did not differ in comparison to the control subjects. In the FH group the mean Lp(a) level of 54.8±62.1 mg.dL^{-1} (range 3.4-336.0 mg.dL^{-1}, median 25.4 mg.dL^{-1}) was higher than expected in normolipidaemic volunteers [19]. All FH patients were treated with lipid lowering drugs for a mean

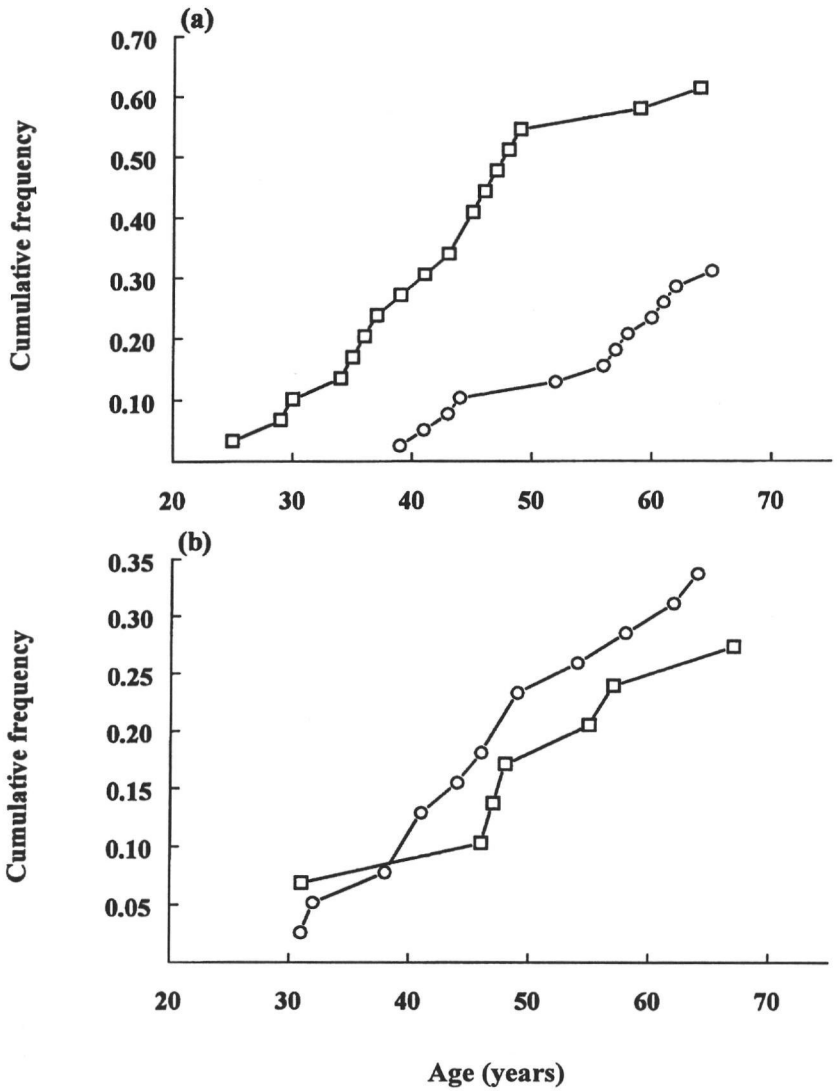

Fig. 2 (a) The cumulative frequency of coronary artery disease (CAD) in patients with familial hypercholesterolaemia as a function of the age onset. (b) The cumulative frequency of peripheral vascular disease (PVD) in patients with familial hypercholesterolaemia as a function of the age upon which PVD was detected (□ = males, ○ = females)

Table 2. Basal and treatment levels of lipids and lipoproteins, type and duration of lipid lowering treatment of all patients with familial hypercholesterolaemia (FH_{TOTAL}, n=68), with (FH_{PVD+}, n=21) and without (FH_{PVD-}, n=47) peripheral vascular disease, and of normocholesterolemic control subjects (n=27)[a]

Group		Tot.chol ($mmol.L^{-1}$)	HDL-chol ($mmol.L^{-1}$)	LDL-chol ($mmol.L^{-1}$)	Triglycerides ($mmol.L^{-1}$)	Lp(a) ($mg.dL^{-1}$)	Medication (inhibitor/resin)	Treatment (years)
FH_{TOTAL}	basal	11.11±1.84*	1.09±0.32*	9.19±2.03*	1.77±0.73	-	-	-
	treatment	7.35±1.30*	1.18±0.33*	5.51±1.35*	1.50±0.62	54.8±62.1	61/28	5.5±3.7
FH_{PVD+}	basal	11.37±1.79	1.17±0.27	8.73±2.08	1.84±0.42	-	-	-
	treatment	7.51±1.32	1.26±0.34	5.54±1.39	1.63±0.67	50.3±49.0	21/5	5.2±3.2
FH_{PVD-}	basal	10.93±1.85	1.06±0.33	9.19±2.09	1.75±0.82	-	-	-
	treatment	7.28±1.29	1.15±0.32	5.49±1.33	1.45±0.61	57.0±68.1	40/23	5.6±3.9
Controls		5.79±1.47	1.41±0.40	3.75±1.30	1.61±1.25	-	-	-

*[a]Values are means±SD; H(L)DL-chol, high (low)-density lipoprotein cholesterol; Lp(a), Lipoprotein(a); inhibitor/resin, number of patients on HMG-CoA reductase inhibitors and/or resins, respectively; differences versus control subjects: *$P \leq 0.001$; no significant differences were found between FH_{PVD+} and FH_{PVD-}*

period of 5.5±3.7 years (range 0.6-15 years, median 4.7 years); 61 of 68 (89.7%) patients were treated with a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (simvastatin or pravastatin), 28 out of 68 (41.2%) used a resin (cholestyramine or colestipol), and 21 out of 68 (30.9%) a combination of both groups of drugs (Table 2). During lipid-lowering treatment, the levels of total cholesterol, LDL cholesterol and HDL cholesterol in the FH group were still significantly different in comparison with the control subjects (Table 2).

Peripheral vascular disease

Vascular measurements were performed during lipid lowering treatment. The ankle/arm pressure ratios at rest were 1.03±0.12 and 1.08±0.11 (P=0.05) and 0.89±0.13 and

Table 3. Prevalence of haemodynamically significant peripheral vascular disease (PVD) and Doppler spectrum waveform characteristics at rest and after reactive hyperaemia in patients with familial hypercholesterolaemia in comparison to normocholesterolaemic control subjects[a]

	Familial hypercholesterolaemia			
	PVD_{AI+}	PVD_{FT+}	PVD-	Controls¶
Number (%)	5 (7.4)	16 (23.5)	47 (69.1)	26 (96.3)
At rest				
AAI	0.92±0.15*	0.93±0.12	1.05±0.10	1.09±0.12
F_{max} (Hz)	4087±934*	7177±2645	6909±2077	6924±2496
F_{min} (Hz)	-1314±991	-1622±405	-1857±626	-1827±630
SL_{down} ($m.s^{-2}$)	-21±7*	-36±8	-33±13	-35±14
T_{max} (ms)	129±24#	102±17	101±16	100±24
PI	4.03±1.96	5.01±1.49	5.00±1.27	4.96±1.64
After hyperaemia				
AAI	0.75±0.17#	0.77±0.07	0.95±.011	0.96±0.08
T_{max} (ms)	133±54	100±39	105±36	98±32
RI	0.60±0.10	0.65±0.08	0.68±0.10	0.68±0.08

*[a]Values are means±SD or numbers (percentages); PVD_{AI+}, aorto-iliac pathology; PVD_{FT+}, femoro-tibial pathology; PVD-, no peripheral vascular disease; AAI, ankle-arm pressure ratio; for explanation of Doppler parameters see 'Materials and methods' section. ¶One patient with PVD is excluded. Differences versus FH_{PVD-} and control subjects: * $0.01 \leq P < 0.05$; # $0.001 \leq P < 0.01$*

0.95±0.09 (P=0.03) after reactive hyperaemia for all the FH patients and control subjects, respectively. One FH patient was known to suffer from intermittent claudication (a female; first symptoms at 38 years of age). Twenty-one out of 68 (30.9%, eight males and 13 females) FH patients had a pathological low ankle/arm systolic blood pressure ratio bilaterally or unilaterally (Table 3). In 39 out of 136 (28.6%) limbs, the pressure index was abnormal, so three patients exhibited a low ratio in only one leg. The patients with PVD were distributed equally within the different age groups (Fig. 1). The youngest patients with haemodynamically significant PVD were 31 years old (n=3) and most patients were found in the 40-49 year-old age cohort. The mean age at which PVD was found for the total group, namely 47.2±11.0 years (range 31-64 years, median 47 years), was not different for men and women (47.8±12.4 and 46.8±10.6 years, respectively). The cumulative frequency of PVD as a function of the age of detection is shown in Figure 2(b). The total frequency in the male population of 27.6% (8/29) was not significantly different when compared with 33.3% (13/39) in females.

Peripheral vascular disease was predominantly found in the femoro-tibial region in 16 out of 21 (76.2%) patients (Table 3). Eight of these 16 patients with a decreased ankle/arm index of <0.90 and a normal Doppler spectrum were detected by measurements at rest, and the remaining eight patients had a decrease in the pressure index of ≥0.20 during reactive hyperaemia. Peripheral vascular disease in the aorto-iliac vessels, showing an abnormal Doppler spectrum of the femoral artery, was found in five out of 21 (23.8%) FH patients with a decreased ankle/arm index. Some parameters of the Doppler spectrum waveforms are shown in Table 3. Post-stenotic waveforms in patients with aorto-iliac pathology are characterized by a decreased maximum frequency shift (F_{max}) and resistance index (RI), and an increase in the duration of the acceleration phase (T_{max}) [16,20]. Only one out of 27 (3.7%, a female) control subjects had significant changes in the femoro-tibial tract, showing bilaterally pathological pressure indexes at rest and normal Doppler spectra. Comparison of the prevalence figures of PVD in the FH group and the control subjects showed significant differences, either expressed per patient (P=0.01) or per limb (P<0.001).

Characteristics of the FH patients with and without PVD are shown in Table 1 and 2. No differences were found with regard to gender, age, body mass index, smoking habits, alcohol consumption, lipids levels (basal or during lipid lowering treatment), Lp(a) concentrations, and the duration and the type of administered lipid lowering drugs. Hypertension in the medical history of the FH patients with PVD was found more often. Only the mean arterial blood pressure before the vascular measurements was significantly higher in comparison with the patients without PVD: 104±9 mmHg versus 93±11 mmHg (P<0.001), respectively. A tendency was observed in the FH patients with PVD to have more clinical symptoms of CAD (angina pectoris) than in those without PVD. Only ECG-changes at rest or during exercise tests had a significantly greater presence in the group with PVD: 71.4% versus 31.9% (P=0.006), respec-

tively. Thirty out of 68 (44.1%) of the total group of FH patients had CAD, 15 (50%) of whom had abnormal pressure indexes, whereas only six out of 38 (19%) patients without CAD had PVD. Therefore, the relative risk of finding haemodynamically significant PVD of the lower extremities in FH patients with signs or symptoms of CAD compared with FH patients without CAD is 3.17 (95% confidence limits 1.40-7.17).

Discussion

Peripheral vascular disease in population studies is unequivocally associated with dyslipoproteinaemia [21,22]. Our study addressed the question of the prevalence of PVD given the presence of well-defined FH. In FH populations, PVD has been reported in only 1.5% of cases as the first manifestation of vascular disease, whereas symptoms of CAD are the first feature in 94% [7]. In a prospective cohort study in subjects with heterozygous FH in England and Wales, a prevalence of intermittent claudication in the 40 to 59 year-old age group has been found in 8.8 and 9.7% of men and women, respectively [23]. In the present study, using non-invasive measurements, haemodynamically significant lesions were present in approximately one out of three asymptomatic FH patients, and PVD was the first atherosclerotic manifestation in 19%. Of course, studies based on the detection of symptoms yield lower prevalence figures than those using non-invasive measurements, whereas the latter show intermediate figures when compared to studies using invasive angiographic methods [22,24,25].

The haemodynamically significant PVD prevalence figure of 31% seems lower than has been shown by others using pressure ratio index measurements [10,11]. However, a higher cut-off point for a pathological ankle/arm pressure ratio index <0.97 has been used in these studies, and this may have overestimated the presence of PVD, since the use of a cut-off point of <0.90 appears to be more predictive for significant obstruction [26].

In the past few years, there has been an increasing use of B-mode ultrasound in the assessment of atherosclerotic changes by measuring the intima-media thickness in the carotid and femoral artery [27,28]. This method may show early atherosclerotic manifestations of the intima-media complex which will not interfere with flow in contrast with Doppler spectrum analysis. However, haemodynamically significant changes in blood flow are comparable for both methods: in a recent study using B-mode ultrasound assessment in patients with heterozygous FH, plaques causing haemodynamically significant changes in blood flow in the femoral artery were found in 14 out of 36 (39%) patients, a figure that is comparable to that found in the FH population of our study [28].

There is considerable variability in the clinical manifestation of patients with heterozygous FH [1]. The phenotypic expression may be influenced not only by differ-

ences in the underlying mutation, the apolipoprotein E phenotype, lipid and lipoprotein levels, and susceptibility to oxidative modification of LDL, but also by general risk factors such as gender, obesity, hypertension, and smoking [5,6,29-32]. In our study, no significant differences between the FH patients with and without PVD were found with regard to lipid and lipoprotein levels and general risk factors, with the exception of a higher blood pressure before the vascular measurements. Therefore, moderate hypertension may have increased the risk of development of PVD in the group of FH patients we studied.

The present study also showed that PVD was mainly found in the femoro-tibial segments and less in the aorto-iliac vessels. Using strain-gauge plethysmography, Koivosto *et al.* also demonstrated the predominant affection of the proximal lower limb arteries in FH [10]. However, Rubba *et al.* have shown predominantly aorto-iliac pathology in FH using Doppler spectrum analysis of the common femoral artery at rest [33]. Using ultrasound methods as described in our study, only haemodynamically significant lesions (stenosis ≥50%) causing a decrease in arterial pressure or flow are documented, and addition of analyses during hyperaemia invariably increases the sensitivity of this method [16]. Therefore, the ultrasound measurements at rest of previous studies may have underestimated the presence of PVD. Since Doppler spectrum analysis at rest ánd during reactive hyperaemia accurately demonstrates the presence of aorto-iliac disease, even if atherosclerotic lesions of the femoral artery in multilevel occlusive disease exist, the presence of haemodynamically significant aorto-iliac pathology is probably not underestimated in our study [34]. Therefore, the primary predilection side of PVD in FH seems to be the proximal lower limb arteries or the femoral-popliteal segment. Generally, a comparison between the age of onset of symptoms of CAD and the age at which PVD was detected in this study is debatable. Still, CAD and PVD can be found only when haemodynamically significant atherosclerotic lesions are present. Indeed, manifestations of CAD can occur coexistent with PVD in FH patients [5,11,13,25]. Our study showed that some patients develop CAD and PVD as early as the third decade of life, and that the mean age of onset of CAD was comparable to the mean age upon which PVD was detected (46 versus 47 years, respectively). Moreover, 50% of the patients with established CAD also exhibited PVD, whereas only 19% of the patients without CAD showed PVD. Therefore these data show that there is a three-fold relative risk for (asymptomatic) PVD once a patient with heterozygous FH exhibits signs or symptoms of CAD.

In conclusion, haemodynamically significant PVD in patients with heterozygous FH is much more prevalent than is expected on clinical grounds, especially in those with CAD. Males and females are equally involved, and the preferential localization in the femoro-popliteal vessels was not related to the lipoprotein levels or general risk factors in this small group of patients, with the exception of hypertension.

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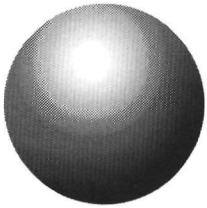

Chapter

Nine

The effect of LDL-apheresis on peripheral vascular disease in hypercholesterolemic patients with coronary artery disease

Provisionally accepted for publication as:

The effect of LDL-apheresis on peripheral vascular disease in hypercholesterolemic patients with coronary artery disease

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Objective: To compare the effect of conventional versus more aggressive lipid lowering with LDL-apheresis on changes in peripheral atherosclerotic disease.
Design: A prospective, open, randomized, single center study.
Setting: One university hospital.
Patients: 42 men with primary hypercholesterolemia and extensive coronary atherosclerosis.
Intervention: Biweekly low density lipoprotein (LDL) apheresis plus simvastatin (40 mg/day) or simvastatin treatment (40 mg/day) alone for a period of 2 years.
Measurements: ankle-arm systolic blood pressure index combined with Doppler spectrum analysis of the femoral artery at rest and during reactive hyperemia, and mean intima-media thickness (IMT) of near and far wall segments of the carotid artery, assessed yearly.
Results: Baseline (mean ± SD) LDL cholesterol was reduced 63% from 7.8 to 3.0 $mmol.L^{-1}$ in the apheresis group versus 47% from 7.9 to 4.1 $mmol.L^{-1}$ in the medication group. The number of hemodynamic stenoses in the aortotibial tract in the apheresis group reduced from 17 to 13 ($p=0.36$) and increased from 11 to 23 ($p=0.008$) in the medication group. Mean IMT in the apheresis group regressed from 0.91±0.38 to 0.85±0.29 mm ($n=11$; $p=0.001$) but thickened in the medication group from 0.88±0.30 to 0.93±0.40 mm ($n=11$; $p=0.002$). With multiple regression analysis changes in carotid IMT were mainly explained by changes in lipoprotein(a) and apolipoprotein A1 levels, and aortotibial changes by mean in-trial apolipoprotein B concentrations and changes in total cholesterol and lipoprotein(a).
Conclusions: Aggressive lipid lowering with medication and LDL-apheresis induced regression of early atherosclerotic lesions in the carotid artery and prevented increase of the number of hemodynamic stenosis in the lower limbs, whereas conventional treatment did not prevent progression of carotid and aortotibial vascular disease.

Introduction

Until today intensive lipid lowering in men with established coronary artery disease (CAD) using HMG-CoA reductase inhibitors is the most effective means in terms of slowing or arrest of progression of coronary atherosclerosis and consequently reducing the number of clinical events [1,2]. Epidemiologic studies have shown parallel, age-related trends of atherosclerotic lesions in the abdominal aorta, carotid, and coronary arteries [3-5]. In several clinical trials on plasma lipid regulation and risk factor analysis measurements of peripheral vascular disease (PVD) have been performed [6-16]. Most of these studies have shown a slowing of progression of either carotid artery intima-media thickness (IMT) or the extent of femoral atherosclerosis during lipid lowering treatment.

Continuous LDL-apheresis using dextran sulfate cellulose columns selectively removes apolipoprotein B-containing lipoproteins from plasma [17]. The performance of regular apheresis permits the achievement of lower levels of low density lipoprotein (LDL) cholesterol which is usually not possible to attain with drug therapy alone [18]. The application of this method may offer opportunities in the prevention of progression, or even inducing regression of atherosclerosis in selected patients with a primary hyperlipidemia and established CAD or PVD [10,19-22]. The LDL-Apheresis Atherosclerosis Regression Study (LAARS) was designed as a 2-years, prospective, open, randomized, single-center study in men with primary hypercholesterolemia and extensive CAD. The objective was to determine whether more aggressive LDL cholesterol lowering with biweekly LDL-apheresis plus simvastatin, a potent HMG-CoA reductase inhibitor [23,24], exerts better an anti-atherosclerotic effect than lipid lowering to more conventional cholesterol levels with simvastatin alone.

Ultrasound techniques are important surrogate variables for clinical end-points in quantitative measurements of atherosclerotic manifestations [25]. The ankle/arm systolic pressure ratio at rest and during hyperemia is a reproducible method to determine the presence of arterial insufficiency in the lower limbs [26,27]. To differentiate between hemodynamic significant stenotic segments located in the aortoiliac or the femorotibial tract, measurement of this pressure ratio should be combined with the assessment of blood flow velocities in the common femoral artery obtained by Doppler spectrum analyses [28,29]. It has also been found that high-resolution B-mode ultrasonography of the carotid artery is an effective and accurate method in the assessment of atherosclerotic changes of the arterial wall [30-32]. This technique has now been applied in a number of studies and it has been shown that the intima-media thickness (IMT) of the carotid artery reflects generalized atherosclerosis, indicated by its association with coronary and lower extremity atherosclerosis [33-39].

In this article the results from LAARS of the sequential assessments of hemodynamic stenosis in the lower extremities and the carotid artery IMT are presented

and related to the lipid and lipoproteins levels. The results of the quantitative analysis of sequential coronary angiographies and exercise tolerance tests have been described recently [40].

Materials and Methods

Subjects and treatment

The design of the trial has been described previously [40]. Briefly, eligibility criteria included: a) men aged between 30 and 67 years, b) a mean of two successive serum total cholesterol determinations above 8.0 $mmol.L^{-1}$ or LDL cholesterol above 5.8 $mmol.L^{-1}$, and a mean of two successive fasting serum triglycerides measurements below 5.0 $mmol.L^{-1}$ on a standard lipid lowering diet (American Heart Association step I) without other lipid lowering treatments, and c) extensive coronary atherosclerosis as shown by visual assessment of the baseline coronary angiogram. Excluded were patients with a myocardial infarction or percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG) within the previous 3 months, impaired hepatic (>30% above normal range) or renal function (plasma creatine ≥ 150 $\mu mol.L^{-1}$), hypertension (diastolic blood pressure ≥ 100 mmHg), diabetes mellitus, severe obesity (BMI ≥ 30 $kg.m^{-2}$), hyperhomocysteinemia, homozygous familial hypercholesterolemia, heavy smokers (>10 cigarettes per day), and any secondary hyperlipidemia. During a 2-months run-in period a coronary angiogram and vascular measurements of the aortotibial tract and the carotid artery were performed. Those patients who proved to be eligible were allocated at random to either biweekly LDL-apheresis plus simvastatin (40 mg/day) or simvastatin treatment (40 mg/day) alone. Randomization was stratified for the level of serum total cholesterol and lipoprotein(a) [Lp(a)], age, and CABG status.

LDL-apheresis was performed with an automated system (MA-01 unit, Kanegafuchi Chemical Industry Co. Ltd., Osaka, Japan) with 2 small-sized dextran sulfate cellulose columns (Liposorber®), selectively absorbing apolipoprotein (apo) B-containing particles. A volume of 5000 mL (approximately 1.5 plasma volume) was treated per session. The combination therapy of LDL-apheresis and simvastatin can be expected to slow down the post-apheresis rebound in serum cholesterol, which permits prolongation of the intervals between the apheresis procedures [41,42]. Patients allocated to lipid lowering with drugs attended the outpatients clinics each month; those on LDL-apheresis were seen fortnightly. In both groups a resin in the highest tolerable dose was added to the treatment if (pre-apheresis) serum cholesterol levels in 2 consecutive months remained above 8.0 $mmol.L^{-1}$, since it was considered not appropriate to continue single drug treatment above this level. At each visit the patients were subjected to a brief physical examination and dietary instruction was repeated frequently.

Lipids, lipoproteins, and other measurements

In the apheresis group lipids and lipoproteins were measured biweekly, before and immediately after each LDL-apheresis, and in the medication group monthly. Apo A1, apo B, and Lp(a) were measured bimonthly. Serum total cholesterol and fasting triglycerides were determined enzymatically (CHOD-PAP, no. 237574, Boehringer Mannheim GmbH, FRG and Sera-PAK, no. 6639, Miles, Italy, respectively). High density lipoprotein (HDL) cholesterol was determined using the polyethylene glycol 6000 precipitation method [43]. LDL cholesterol was calculated by subtraction. Samples for apo A1, apo B, and Lp(a) were stored at -80°C, and determined at the end of the study. Apo A1 and apo B were quantified in serum by immuno-nephelometry [44]. Lp(a) was measured by a specific radioimmunoassay [apolipoprotein(a) RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden].

The selective removal of apo B-containing lipoproteins with LDL-apheresis causes sawtooth-like alterations in lipoprotein concentrations [45]. The increase of lipoprotein levels after the treatment can be explained by first order kinetics [22,46]. Therefore, time-averaged concentrations (C_{AVG}) or interval means of total cholesterol, LDL cholesterol, apo B, and Lp(a) were calculated by applying a formula derived from rebound curves which were constructed for each patient at one occasion: $C_{AVG}=C_{MIN}+0.73(C_{MAX}-C_{MIN})$, where C_{MAX} is the pre-treatment level and C_{MIN} the levels immediately after apheresis. For serum triglycerides and HDL cholesterol only pretreatment levels were used in the analysis, because triglycerides reach pretreatment levels within 1 to 2 days after apheresis and HDL cholesterol is not influenced by LDL-apheresis. Apheresis produced an acute reduction of 35% in fibrinogen levels, returning to pre-treatment concentrations between 2 and 7 days (n=11, data not shown). Fibrinogen concentrations at baseline did not differ between both groups.

Ankle/arm systolic pressure ratio measurements and Doppler spectrum analyses

The analysis of blood flow velocities in the common femoral artery obtained by Doppler spectrum measurements has proven to be an accurate, non-invasive, easy-to-perform screening-test to assess functionally and hemodynamically significant stenosis in the aortotibial tract [28,29]. Measurements were performed at baseline, after 1 year, and after 2 years of treatment. In one session blood flow velocities in the common femoral arteries and ankle/arm systolic blood pressure ratios of both limbs were recorded, both at rest and during reactive hyperemia. The performance of Doppler spectrum analysis during reactive hyperemia has shown to enhance the sensitivity for detection of aortoiliac pathology [47,48]. Doppler signals were obtained from the common femoral artery with an 8 MHz bidirectional continuous-wave Doppler apparatus (Angiodine 2, D.M.S. Montpelier, France) by placing the probe just below the inguinal ligament, as described previously [49]. Reactive hyperemia was induced by thigh cuff compression for 5 minutes at a pressure of at least 50 mmHg above the systolic arterial thigh pres-

sure. Doppler spectra during reactive hyperemia were obtained approximately 15 seconds after relief of the thigh compression. Maximum-frequency waveforms were calculated from the spectra and several parameters were used that describe the shape of the waveforms [50]. Based on a combination of 6 of these Doppler parameters [duration of the acceleration phase (Tmax), slope of the deceleration phase (SLdown), maximum frequency of receding curve (Fmin), and pulsatility index (PI=Fmax-Fmin/Fmean) of the at-rest spectra and duration of the acceleration phase (Tmax) and resistance index (RI=Fmax-Fdia/Fmax) of the spectra during reactive hyperemia] the presence of hemodynamic significant aortoiliac pathology was assessed, and could be expressed as a continuous variable [47]. The same Doppler probe was used to determine the ankle/arm pressure index, using the systolic radial artery pressure and the highest pressure in either the dorsalis pedis or posterior tibial artery. Hemodynamic significant vascular disease was defined as a ankle/arm pressure index at rest <0.90 and/or a decrease of the pressure index during reactive hyperemia ≥ 0.20.

For evaluation of the results patients were categorized as follows. Patients with at least one new hemodynamic significant lesion in the aortoiliac or femorotibial tract of either sides were defined as worsened. Improved were patients with a reduced number of lesions and stable patients showed no difference in number or location of hemodynamic stenosis. If a patient had to undergo an angioplasty procedure (PTA) or vascular surgery for progressive claudication, he was considered to be worsened irrespective of the outcome of the test.

Measurements of carotid artery intima-media thickness (IMT)

Two-sided high resolution B-mode ultrasound examinations of the carotid arteries were performed at baseline, after 1 year, and after 2 years of treatment. Baseline measurements could not be done in the first patients of the study since the method was not available at the start of the study. Therefore, three sequential assessments, including baseline, of 11 patients in both groups could be evaluated and two measurements in the remaining men. Scannings were done with a duplex apparatus (ACUSON 128XP, Cardia-Acuson B.V., Mountain View, CA, USA) equipped with a 7 MHz L7384 linear array / 5.0 MHz Doppler transducer combination. In order to enhance reproducibility, longitudinal scans were performed from a fixed latero-lateral angle, using the dilation of the common carotid artery and the flow divider in the carotid artery as anatomical landmarks. Greatest care was taken to obtain a double-line pattern both from the near and far wall. The IMT was defined as the distance (mm) between lumen-intima and media-adventitia interfaces of the arterial far wall. Although, near and far wall do not represent identical anatomical correlates [51], combined measurements can be used in an intervention study since rates of progression between both wall measurements do not differ [52]. Moreover, the inclusion of the near wall reduces the variability of the measurement. If a plaque was located where IMT measurement should be performed,

the plaque thickness was included in the IMT value. The ultrasound images of the arterial segments were stored in real-time on a S-VHS videotape. Follow-up scans from each patient were made by the same sonographer, who was specially trained. At the end of the study off-line analysis of the IMT was performed at the CORE Laboratory of the Interuniversity Cardiology Institute (ICIN, Utrecht, the Netherlands) by professional readers blinded for the treatment. The IMT of the common carotid artery (the segment 10 mm proximal to the dilation), the bifurcation (the segment between the flowdivider and the dilation), and the internal carotid artery (the segment 10 mm distal to the flowdivider) was measured by a semi-automated contour detection program. For each subject a mean IMT from 12 combined arterial sections (2 sides, 2 arterial walls, 3 segments) per patient was taken as a measure for wall thickness. Effects on individual carotid segments were also investigated. To estimate the intraobserver variability 10 randomly selected patients were invited for a repeated IMT assessment 7 days after the original one. The coefficient of variation, computed as the proportion of the standard deviation of the overall mean, was 11.5% (intraobserver error 0.10 mm).

Table 1. Baseline characteristics

	Apheresis (n=21)	Medication only (n=21)	P*
Age [years]	50.2±9.6	53.9±8.7	0.43
Weight [kg]	81.5±9.7	80.8±8.6	0.88
BMI [$kg.m^{-2}$]	26.6±2.0	26.2±2.0	0.64
Blood pressure [mmHg]			
systolic	129.3±17.3	126.3±18.1	0.56
diastolic	78.2±8.9	76.5±9.0	0.63
Current smoking	3 (14.3)	4 (19.0)	1.00
Myocardial infarction	16 (76.2)	18 (85.7)	0.69
CABG	10 (47.6)	10 (47.6)	0.75
PTCA	2 (9.5)	5 (23.8)	0.41
Hypertension	2 (9.5)	5 (23.8)	0.41
Stroke	1 (4.8)	3 (14.3)	0.60
Claudication	3 (14.3)	5 (23.8)	0.69

*Values indicate numbers (percentages) or means ± SD; *, t-test or chi^2-test where appropriate. BMI, body mass index; CABG, coronary artery bypass graft; PTCA, percutaneous transluminal coronary angioplasty*

Table 2. Changes in lipids and lipoproteins: baseline and treatment levels

	Apheresis (n=21)			Medication only (n=21)			Difference
	basal	interval mean	% change	basal	mean	% change	*P*
Total cholesterol ($mmol.L^{-1}$)	9.72±1.84	4.63±1.18	-52.9±6.6	9.85±2.17	5.95±1.60	-39.5±7.7	0.005
Triglycerides ($mmol.L^{-1}$)	2.32±1.03	1.83±0.76	-17.4±24.4	2.64±1.33	1.84±0.89	-26.5±20.3	0.38
LDL cholesterol ($mmol.L^{-1}$)	7.78±1.86	2.95±1.13	-62.9±8.3	7.85±2.34	4.13±1.58	-47.4±8.1	0.01
HDL cholesterol ($mmol.L^{-1}$)	0.93±0.18	1.09±0.20	+17.7±13.6	0.92±0.19	1.05±0.22	+13.7±10.9	0.23
Lipoprotein(a) ($mg.dL^{-1}$)	57.0±63.9	44.5±54.3	-18.6±18.0	38.4±39.7	44.5±45.7	+14.9±16.3	0.02
Apolipoprotein B ($g.L^{-1}$)	2.59±0.47	1.32±0.35	-49.0±7.6	2.60±0.61	1.74±0.47	-31.0±17.0	0.003
Apolipoprotein A1 ($g.L^{-1}$)	1.43±0.29	1.34±0.17	-5.3±13.0	1.46±0.38	1.33±0.21	-5.4±16.8	0.89

Data represent means ± SD. Apheresis group: 52 measurements per patient. Medication group: 26 measurements per patient. Interval mean, time-averaged levels, calculated by estimation of the area under the rebound curve (see text); % change, difference between basal and mean levels; P, p-values of differences between interval mean in the apheresis group and mean levels in the medication group (t-test or Mann-Whitney U-test where appropriate). To convert values for total cholesterol to mg/dL, multiply by 38.67, and to convert values for triglycerides to mg/dL, multiply by 88.57

Statistical analysis

The sample size of the study was limited for logistic reasons to approximately 40 patients. Sample size calculations were based on expected changes in the coronary arteries [40]. Analyses were performed with procedures available in the statistical package for social sciences (SPSS Inc., Chicago, IL.), using Student's *t*-test, multivariate ANOVA (with correction for repeated measures) for normally distributed data, or Mann-Whitney U-test for differences in means of not normally distributed data. Differences in proportions were analyzed with the (Yates') corrected chi^2-test, and analyses for trends in proportions were done with the extended Mantel-Haenszel chi^2-test. A two-sided Fisher's exact test was employed when the total number of cases was less than 15. For measures of agreement the Pearson product-moment correlation coefficient was used. Stepwise least-squares multivariate regression analysis was performed using a maximal p-value in the F-test of 0.05 before a variable could be added and a minimal tolerance (collinearity) of 0.01. Analyses were based on randomization assignment (intention-to-treat); data forgoing peripheral vascular surgery or angioplasty were used as end-point measurements. A two-sided p-value of less than 0.05 was considered to be significant. Results are expressed as means ± SD, unless otherwise indicated.

Results

Baseline characteristics

Twenty-one men were enrolled in each group of whom the baseline characteristics are shown in Table 1. In both groups 16 patients were heterozygous for familial hypercholesterolemia (76% of the study population). Risk factors for atherosclerosis were equally distributed. All patients had severe angiographically demonstrated coronary atherosclerosis. By the criterion of a stenosis of ≥50% being considered significant, 17 of 21 men in the apheresis group, and 19 of 21 men in the medication group had 3-vessel disease of the coronary arteries. The other patients had 2-vessel coronary artery disease. A previous history of stroke or intermittent claudication was present in 4 men of the apheresis group versus 8 in the medication group. None of the patients were hypertensive at the start of the study. Basal cholesterol levels were high, and predominant elevation of apo B-containing lipoproteins were found in agreement with the inclusion criteria (Table 2). Lp(a) levels showed a skewed distribution with median baseline levels of 28.8 and 19.8 $mg.dL^{-1}$ in the apheresis and medication group, respectively. The treatment groups were well balanced and no significant differences were found with respect to baseline characteristics and baseline lipid and lipoprotein concentrations.

Table 3. Number of sides with hemodynamic significant stenoses in the aortoiliac and femorotibial tract as measured with Doppler spectrum analyses of the femoral artery and ankle-arm systolic blood pressure ratios at rest and after reactive hyperemia, at baseline, after 1 year, and after 2 years of treatment

	Apheresis (n=40)				Medication only (n=42)				Difference
Location	Test 1	Test 2	Test 3	P_A	Test 1	Test 2	Test 3	P_M	P_D
Aortoiliac	7	4	2	0.07	7	8	11	0.28	0.003
Femorotibial	15	13	11	0.34	6	14	18	0.004	<0.001
Whole tract	17	16	13	0.36	11	18	23	0.008	0.002

Test 1, 2, and 3: assessments at baseline, after 1 year, and after 2 years of treatment, respectively; P_A and P_M, p-values of trends in proportions in Apheresis (A) group and Medication (M) group; P_D, p-value of difference (D) in trends between both treatments (extended Mantel-Haenszel chi^2-test)

Table 4. Ankle-arm systolic blood pressure ratios and some Doppler spectrum waveform characteristics at rest and after reactive hyperemia at baseline, after 1 year, and after 2 years of treatment

	Apheresis (n=40)				Medication only (n=42)				Difference
	Test 1	Test 2	Test 3	P_A	Test 1	Test 2	Test 3	P_M	P_D
At rest									
AAI	1.07±0.11	1.05±0.12	1.07±0.13	0.48	1.02±0.14	0.99±0.18	0.98±0.18	0.03	0.34
Fmax (Hz)	5950±1696	5927±1693	5934±1375	0.99	5529±1490	5433±1697	5409±2051	0.90	0.97
Fmin (Hz)	-1962±620	-1853±437	-1985±406	0.18	-1801±609	-1584±584	-1598±540	0.02	0.28
Sldown ($m.s^{-2}$)	-33±10	-29±10	-31±9	0.05	-29±8	-26±9	-27±9	0.25	0.74
Tmax (ms)	101±15	98±16	96±13	0.32	112±26	114±20	115±23	0.61	0.25
PI	5.00±0.99	5.25±1.36	5.83±1.14	0.001	5.02±1.22	5.00±1.31	4.90±0.97	0.84	0.005
After hyperemia									
AAI	0.87±0.17	0.89±0.17	0.90±0.17	0.64	0.86±0.22	0.84±0.20	0.84±0.22	0.55	0.39
Tmax (ms)	98±27	95±30	96±15	0.86	102±32	113±44	114±43	0.98	0.90
RI	0.59±0.07	0.60±0.07	0.61±0.10	0.19	0.62±0.09	0.61±0.08	0.59±0.10	0.19	0.04

Data represent means ± SD. AAI, ankle-arm systolic blood pressure ratio; for explanation of Doppler parameters see Materials and Methods section; Test 1, 2, and 3: assessments at baseline, after 1 year, and after 2 years of treatment, respectively; P_A, P_M, and P_D, P-values in Apheresis (A) group, Medication (M) group, and of the difference (D) between both groups, respectively (multivariate ANOVA)

Clinical events and patient evaluation

Two patients, one in each group, had progressive intermittent claudication. The patient from the apheresis group had to undergo an angioplasty (PTA) of the left common iliac artery at 5 months after the start of the study. In the same patient a coronary artery bypass graft (CABG) procedure had to be performed for unstable angina at 9 months, after which he continued only treatment with simvastatin. In the medication group the other patient with progressive claudication had abdominal aortic graft surgery after 12 months of treatment and another patient had a cerebral transient ischemic attack (TIA) at 11 months. In the apheresis group one patients was lost to follow-up, due to death immediately after coronary surgery for unstable angina within 3 months after the start of the study. So, the results of 20 patients in the apheresis group and 21 in the medication group could be evaluated.

Lipid and lipoprotein profiles

Three patients in the apheresis group and 4 in the medication group received additional resin treatment, 8-24 g cholestyramine per day. LDL-apheresis caused an acute reduction of 62%, 78%, 71%, and 72% of the mean concentrations of total cholesterol, LDL cholesterol, Lp(a) and apo B, respectively. HDL cholesterol levels were not influenced by this procedure. Differences in treatment effects were established by comparison of interval mean concentrations in the apheresis group and mean concentrations in the medication group. During the whole course of the study apo B-containing lipoproteins were significantly lower in the apheresis group compared to the medication group (Table 2). In both groups a comparable increase of HDL cholesterol levels and a decrease of serum triglyceride levels was observed while on treatment with simvastatin (Table 2). The LDL/HDL cholesterol ratio was reduced from 8.4 to 2.7 (-68%) in the apheresis group, versus -54% (from 8.5 to 3.9) in the medication group. The interval mean Lp(a) level was reduced by 19 % in the apheresis group, which was significantly different in comparison with the 15% increased levels found in the medication group (Table 2).

Hemodynamic significant lesions in the aortoiliac and femorotibial tract

At baseline no significant differences in number of patients with stenoses in the whole aortotibial tract were observed between both groups: an abnormal low ankle/arm systolic blood pressure ratio was found in 9 patient in the apheresis group versus 6 in the medication group, on 17 versus 11 sides, respectively (Table 3). The ankle/arm pressure ratios at rest were 1.07±0.11 and 1.02±0.11, respectively, and after reactive hyperemia 0.87±0.17 and 0.86±0.22, respectively (Table 4). An abnormal Doppler spectrum of the femoral artery and a decreased ankle/arm ratio, representing aortoiliac hemodynamic significant lesions, were observed on 7 sides in both groups (Table 3). Femorotibial lesions, as shown by a normal Doppler spectrum and a decreased an-

Table 5. Lipids and lipoproteins of patients showing an increase of the number of hemodynamic significant lesions in the aortoiliac and femorotibial tract versus those showing no change or a reduction of the number of stenosis

	Worsening (n=15)			No change/improvement (n=26)			Difference
	basal	mean in-trial	% change	basal	mean in-trial	% change	*P*
Total cholesterol ($mmol.L^{-1}$)	9.75±2.19	6.11±1.85	-37.8±7.2	9.89±1.92	5.01±1.13	-49.2±7.8	<0.001
Triglycerides ($mmol.L^{-1}$)	2.75±1.24	2.03±1.00	-25.7±14.4	2.49±1.27	1.73±0.71	-25.3±16.1	0.82
LDL cholesterol ($mmol.L^{-1}$)	7.70±2.34	4.17±1.68	-46.6±7.2	7.91±2.07	3.30±1.13	-58.3±9.3	<0.001
HDL cholesterol ($mmol.L^{-1}$)	0.93±0.19	1.04±0.24	+11.0±9.2	0.95±0.19	1.09±0.19	+15.9±12.6	0.23
Lipoprotein(a) ($mg.dL^{-1}$)	44.0±40.9	50.8±48.0	+12.0±16.2	47.3±60.5	41.1±52.4	-12.8±16.3	0.002
Apolipoprotein B ($g.L^{-1}$)	2.65±0.58	1.75±0.48	-32.9±14.8	2.56±0.54	1.40±0.33	-44.7±12.1	0.007
Apolipoprotein A1 ($g.L^{-1}$)	1.50±0.38	1.42±0.27	-2.8±13.7	1.43±0.31	1.38±0.16	-1.0±14.7	0.46

Data represent means ± SD. No differences in basal levels were observed. Mean in-trial concentrations in the apheresis group were calculated by an estimation of the area under the rebound curve (see text). % change, difference between basal and mean levels; P, p-values of differences in % change (Mann-Whitney U-test). To convert values for total cholesterol to mg/dL, multiply by 38.67, and to convert values for triglycerides to mg/dL, multiply by 88.57

Table 6. Mean intima-media thickness (mm) in different segments of the carotid artery as measured with B-mode ultrasonoraphy in a subgroup of 22 men, at baseline, after 1 year, and after 2 years of treatment

	Apheresis (n=11)				Medication only (n=11)				Difference
Location	Test 1	Test 2	Test 3	P_A	Test 1	Test 2	Test 3	P_B	P_D
Bifurcation	1.11±0.54	1.02±0.50	0.99±0.35	0.002	0.92±0.29	0.91±0.28	1.08±0.45	<0.001	<0.001
Common CA	0.81±0.18	0.80±0.18	0.76±0.15	0.004	0.80±0.22	0.78±0.19	0.78±0.22	0.19	0.03
Internal CA	0.83±0.27	0.79±0.20	0.79±0.21	0.37	0.91±0.37	0.91±0.39	0.94±0.45	0.85	0.52
all	0.91±0.38	0.87±0.35	0.85±0.29	0.001	0.88±0.30	0.87±0.35	0.93±0.40	0.002	<0.001

Data represent means ± SD from segments of the bifurcation, the common and internal carotid artery (CA). Test 1, 2, and 3: assessments at baseline, after 1 year, and after 2 years of treatment, respectively; P_A, P_M, and P_D, p-values in Apheresis (A) group, Medication (M) group, and of the difference (D) between both groups, respectively (multivariate ANOVA)

kle/arm pressure ratio, were found in 15 limbs in the apheresis group and in 6 of the medication group (p=0.01). At the end of the study the number of patients with hemodynamic stenoses in the medication group was increased from 6 to 13 (p=0.03). This was predominantly based on a significant increase of femorotibial lesions (Table 3). In the apheresis group a not statistically significant trend was observed towards reduction of the number of patients (from 9 to 7) and sides (aortoiliac and femorotibial) with hemodynamic lesions, causing significant differences in trends between both groups at the end of the study (Table 3).

In general, post-stenotic waveforms in patients with aortoiliac pathology are characterized by a decreased maximum frequency shift (Fmax-Fmin) and resistance index (RI), and an increase in the duration of the acceleration phase (Tmax) [47]. Some characteristics of the Doppler spectrum waveforms, addressing only changes in the aortoiliac tract, are shown in Table 4. Significant differences in trends of the pulsatility index [PI=(Fmax-Fmin)/Fmean] and resistance index [RI=(Fmax-Fdia)/Fmax] between both groups were observed, which is in accordance with the decrease in the apheresis group of aortoiliac hemodynamic significant stenoses and their increase in the medication group. In the apheresis group 18 of 20 (90%) patients showed improvement or no change in the number hemodynamic significant stenoses in the whole aortotibial tract, versus 8 of 21 (38%) patients in the medication group (p=0.002, relative risk 2.36 [95% CI: 1.34 to 4.15]). Categorization of all patients in those who showed increase in the number of lesions versus those who showed no change or a reduction in the number of hemodynamic stenoses demonstrated significant more reduction of total cholesterol, LDL cholesterol, Lp(a) and apo B in the group that did not change or improved (Table 5).

Intima-media thickness (IMT) of the carotid arteries

Baseline concentrations of lipids and lipoproteins of the group of 22 men who had 3 IMT measurements, including baseline, were not different in comparison to the whole study group (data not shown). At baseline the IMT of the bifurcation and the internal carotid were significantly different between both groups (Table 6). However, during the study a gradual significant decrease in mean IMT of all segments was observed in the apheresis group compared to an increase in the medication group (Table 6). These changes were due to significant different trends in IMT of the segments in the common carotid artery and the bifurcation between both groups (Fig. 1). Categorization of all patients who showed reduction or no change of mean IMT (n=11) versus those who showed increased thickening (n=11) again demonstrated significantly more reduction of total cholesterol, LDL cholesterol, Lp(a) and apo B, comparable to the data shown in Table 5, and no differences in HDL cholesterol, serum triglycerides, and apo A1 levels were found (data not shown).

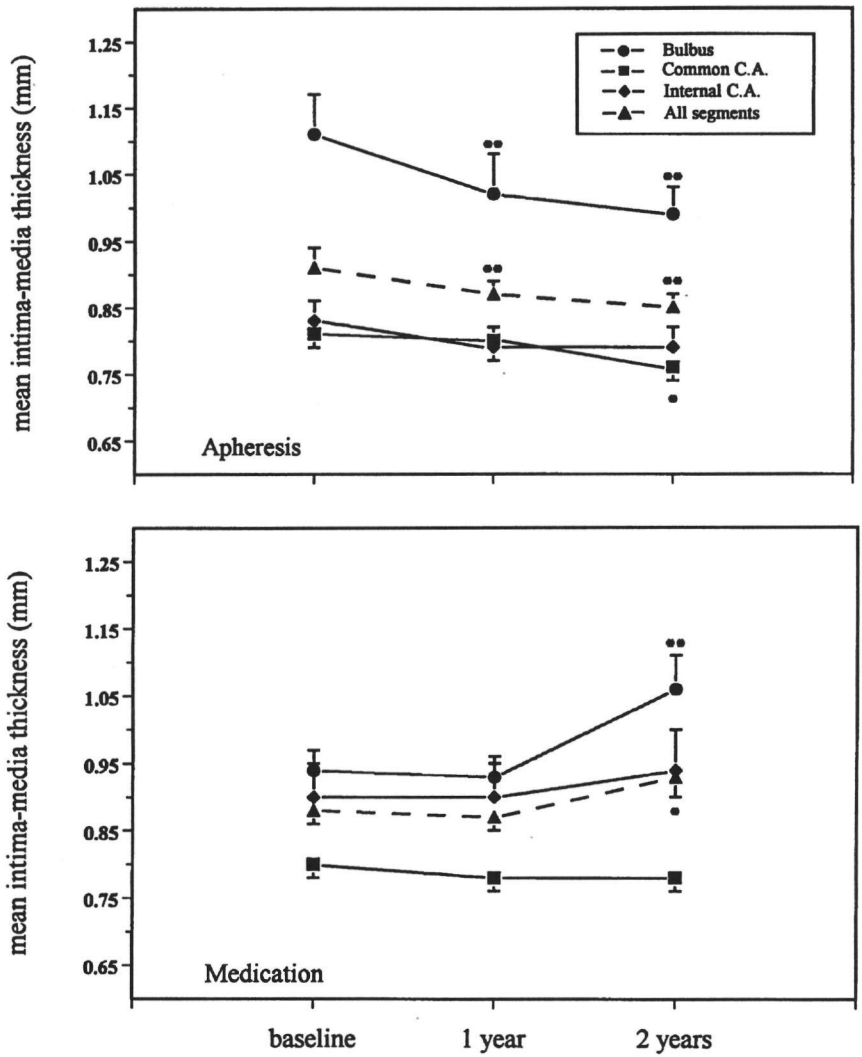

*Fig. 1. Change in mean intima media thickness (mean±SEM) of the bifurcation, the common and internal carotid artery in 11 subjects from the apheresis group versus 11 subjects from the medication group at baseline, after 1, and after 2 years of treatment. (Asterisks denote significant difference versus baseline: *0.01≤P<0.05; **0.001≤P<0.01)*

Correlations

Absolute changes from baseline and mean in-trial concentrations of total cholesterol, LDL cholesterol, and apo B were significantly correlated with worsening or improvement of hemodynamic lesions in the aortotibial tract, indicating that more aggressive lipid lowering was associated with reduction in presence of hemodynamic stenoses

(Table 7). Multiple regression showed that only mean in-trial apo B (variance R^2=0.19, p=0.005), absolute change from baseline of total cholesterol (variance R^2=0.19, p=0.005), and absolute change from baseline of Lp(a) (variance R^2=0.16, p=0.01) explained the changes in the aortotibial tract. Stepwise addition of other variables did not further improve the explained variance significantly (total R^2=0.36, p=0.001). Early atherosclerotic lesions, expressed as changes in mean IMT, were correlated with absolute changes in apo A1 (variance R^2=0.25, p=0.02) and Lp(a) (variance R^2=0.35, p=0.004). Using stepwise multiple regression no other variables further improved the explained variance of the mean IMT significantly (total R^2=0.49, p=0.002). A positive correlation was found between the reduction in mean IMT of the carotid artery and the

Table 7. Correlations between lipids and lipoproteins and changes in hemodynamic significant aortotibial lesions and mean carotid artery intima-media thickness

	Aorto-tibial tract†		Change in IMT‡	
	r	P	r	P
Absolute change from baseline				
Total cholesterol	0.46	0.003	0.25	0.27
LDL cholesterol	0.38	0.01	0.17	0.46
HDL cholesterol	0.20	0.22	0.17	0.45
Lipoprotein(a)	0.44	0.005	0.59	0.004
Apolipoprotein B	0.30	0.06	0.18	0.42
Apolipoprotein A1	0.07	0.66	0.50	0.02
Mean in-trial concentrations				
Total cholesterol	-0.36	0.02	-0.35	0.11
LDL cholesterol	-0.30	0.06	-0.35	0.11
HDL cholesterol	0.12	0.46	0.09	0.71
Lipoprotein(a)	-0.09	0.57	-0.14	0.54
Apolipoprotein B	-0.41	0.009	-0.32	0.14
Apolipoprotein A1	0.12	0.47	0.15	0.50

Data represent Pearson's correlation coefficient (r) and corresponding p-values (P) from pooled data of aortotibial lesions (n=41), expressed as categorial changes (worsened, unchanged, improved)†, and mean intima-media thickness (IMT) of the carotid artery (n=22)‡

reduction of hemodynamic significant stenoses in the aortotibial tract, expressed as categorial changes (worsened, stable, improved) (r=0.44, p=0.048) or as change in a continuous score for the Doppler spectrum waveform (r=0.54, p=0.009) (Fig. 2).

Discussion

LAARS was performed to evaluate whether more aggressive lipid lowering in men with extensive CAD exerts better retardation of the progression of atherosclerosis. The primary outcome measures showed equal arrest of progression of CAD in both groups as assessed by sequential quantitative coronary angiography. However, LDL-apheresis plus simvastatin treatment further reduced the number of early coronary lesions and significantly reduced exercise-induced myocardial ischemia [40]. The present study showed the secondary outcome measures. We demonstrated that aggressive cholesterol lowering prevented increase of the prevalence of hemodynamic stenosis in the aortotibial tract and reduced the mean IMT of the carotid arteries. In other words, 2 years of more aggressive lipid lowering regressed early peripheral atherosclerotic lesions and arrested progression of advanced ones, whereas conventional treatment did

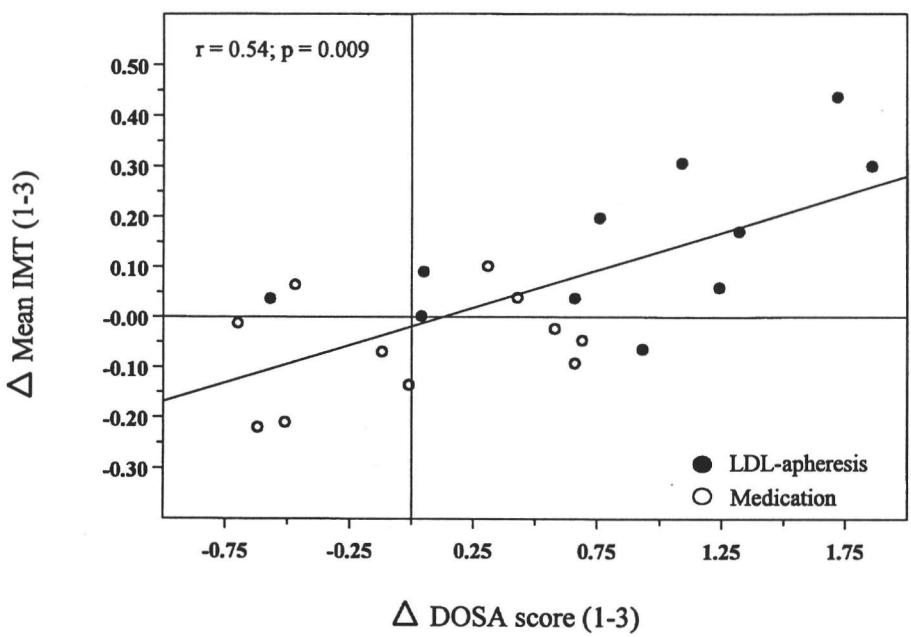

Fig. 2. Correlation between the change (baseline-end of study) in mean intima media thickness (IMT) of the carotid artery and the change (baseline-end of study) in the score of the Doppler spectrum analysis (DOSA) [negative figures depict worsening].

not prevent progression of early and advanced peripheral lesions.
It has been shown that natural occurring progression of CAD is mainly seen in the formation of new lesions and less in growth of preexisting ones, whereas progression of the latter is correlated with high cholesterol levels [53]. Most coronary regression studies showed the greatest benefit in atheroma obstructing >50% of the lumen. However, more recent trials reported also that the formation of new lesions was reduced [54-57]. The present results from LAARS, showing arrest of progression of advanced aortotibial lesions and regression of early carotid lesions, suggest a parallel effect of cholesterol lowering on both CAD and PVD.

The effect of lipid lowering on femoral atherosclerosis has been shown by several studies [7-10]. CLAS was the first placebo-controlled study showing significant reduction of angiographically determined femoral PVD during treatment with colestipol and niacin in men with previous CABG [9]. POSCH showed that fewer subjects in the ileal bypass surgery group developed intermittent claudication and angiographically demonstrated PVD [8]. Only one uncontrolled study showed the beneficial effect of LDL-apheresis in severe atherosclerotic obstruction of the lower extremities in patients refractory to drug treatment [10]. A reduced progression or regression of the carotid artery IMT has been demonstrated in CLAS, MARS, ACAPS, KAPS and PLAC-II [11,12,14-16]. The observed reduction in mean IMT of the common carotid artery after two years in the apheresis group of our study is comparable with the findings of the active treatment groups of CLAS and MARS, which showed a reduction of -0.05±0.06 mm and -0.06±0.11 mm, respectively [11,12]. Remarkable are the observations of the medication group in LAARS which do not seem to benefit from cholesterol lowering. However, the placebo groups of CLAS, MARS, and PLAC-II, all with a mean in-trial LDL cholesterol comparable to our medication group, also showed progression of carotid IMT [11,12,16]. The recently published population-based primary prevention studies ACAPS and KAPS, using lovastatin and pravastatin treatment, respectively, support these results [14,15]. Moreover, ACAPS has shown that reduction of LDL cholesterol to 2.9 $mmol.L^{-1}$ significantly reduced the cardiovascular and all-cause mortality. So, although we analyzed only a small group of subjects in LAARS, our data underline that more aggressive reduction of apo B-containing lipoproteins is necessary to induce regression of atherosclerosis in different peripheral vascular beds.

The accepted indication for LDL-apheresis is resistance to drug treatment in patients with CAD [18]. In LAARS, LDL-apheresis was used as a method to lower LDL cholesterol more aggressively in subjects of whom some were not drug-resistant. Our results confirm the usefulness of extracorporeal therapy in achieving and maintaining low LDL cholesterol levels, as has also been shown recently by Thompson et al [22]. The reduction of baseline LDL cholesterol levels by 63% (95% CI: 59 to 68%) is in keeping with other studies using lipid lowering drugs plus dextran sulfate LDL-apheresis columns [21,58]. On the other hand, LDL reduction in our medication group

by 47% (95% CI: 44 to 51%) may be considered as a good response to simvastatin treatment [23,24] which is probably the result of frequent monitoring of the patients. Since LDL apheresis entails a major commitment for the patient and the medical community, our results affirm the observations from others that hypercholesterolemic patients with established CAD and/or PVD should be treated with a combination of lipid lowering drugs to LDL cholesterol levels < 3.0 $mmol.L^{-1}$ [1]. Consequently, LDL-apheresis should be considered in patients with hypercholesterolemia who are refractory to drug treatment.

It is accepted that the impact of risk factors for the development of atherosclerosis differs among the various arterial segments [59,60]. Cigarette smoking, hypertension, and age have been shown to be more powerful predictors for the presence of PVD than lipids and lipoproteins. The observed differences in effects of risk factor intervention on different arterial segments or between studies may be associated with different visualization technics or different durations of the intervention. Moreover, individual biological susceptibility for risk factors due to lesions of different age and therefore different physicochemical and histological characteristics may also be contributory [16,61]. In LAARS only the effects of changes in lipids and lipoproteins could be analyzed, since no other risk factors were influenced. Changes in the aortotibial tract were explained by absolute reduction of total cholesterol and Lp(a) and by in-trial apo B levels, whereas changes in carotid IMT were explained by absolute change in Lp(a) and apo A1 levels. These findings concerning apolipoproteins and total cholesterol accord with data from population-based and intervention studies [37,62-64]. The influence of Lp(a) as a risk factor should be discussed more in detail with relation to LDL-apheresis. Our study showed a significant reduction of Lp(a) levels in the apheresis group. The role of Lp(a) as an independent risk factor for CAD and cerebrovascular disease, but also for PVD, has been confirmed [65]. However, only recently the FH Regression Study showed that further reduction of Lp(a) levels by LDL-apheresis did not induce more regression of CAD in comparison with drug treatment which lowered LDL cholesterol levels to the same extent [22]. Moreover, in LAARS we also did not observe differences between both treatment groups with regard to changes in coronary anatomy. The present results, however, show a different risk factor relationship between Lp(a) and PVD, especially for early carotid lesions.

One may criticize the results from Doppler spectrum and ankle/arm pressure index measurements by presuming that rheologic changes induced by LDL-apheresis have influenced the results. Indeed, early improvements in the clinical symptoms of claudication and pectoral angina are assumed to be initially induced by correction of rheologic properties of blood [10,66]. Acute reductions by dextran-sulfate adsorption of fibrinogen, factor VIII, and other coagulation factors have been described to last no longer than 1 to 2 days [67]. Still, whole blood and plasma viscosity have been shown to be reduced for a period as long as 1 week [68]. So indeed, it can not be excluded that

improved rheology influenced the measurements of hemodynamic stenosis after 1 year of treatment, while apheresis was still going on. However, at the end of the study the assessments were done 3 to 4 weeks after the last treatment, at the time rheologic changes have disappeared.

In conclusion, the results of LAARS support the evidence that aggressive lipid lowering is beneficial in patients with hypercholesterolemia and coronary atherosclerosis. Combined LDL-apheresis and cholesterol lowering drugs in patients with high risk for cardiovascular events induced regression of the IMT in the carotid arteries and arrested progression of hemodynamic significant stenosis in the aortotibial tract. These findings are consistent with the concept that atherosclerosis is a generalized condition, although the impact of various risk factor interventions may be different.

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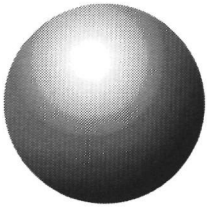

Chapter

Ten

Pregnancy in a patient with homozygous familial hypercholesterolemia treated with long-term low-density lipoprotein apheresis

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Pregnancy in a patient with homozygous familial hypercholesterolemia treated with long-term low-density lipoprotein apheresis

A.A. Kroon, D.W. Swinkels, P.W.J. van Dongen, A.F.H. Stalenhoef

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The pregnancy and delivery of a subject with homozygous familial hypercholesterolemia (FH) and coronary artery disease (CAD) were monitored closely for signs of maternal and fetal distress. Biweekly treatment with low-density lipoprotein (LDL) apheresis using dextran-sulfate cellulose columns was continued throughout the pregnancy, and lipid and lipoprotein levels were evaluated. During the course of the pregnancy and delivery, no signs of maternal coronary insufficiency developed. Serial ultrasonographic measurements of fetal growth indices and the blood flow velocity waveforms (FVWs) of the uterine and umbilical artery did not reveal any sign of fetal growth retardation or insufficiency of the uteroplacental circulation, respectively. During pregnancy, time-averaged concentrations of serum total cholesterol (TC), LDL cholesterol (LDL-C), apolipoprotein (apo) B, and lipoprotein(a) [Lp(a)] showed a gradual decline. Notwithstanding LDL-apheresis, a gradual twofold increase of serum triglyceride (TG) levels was found. In the second and third trimester, high-density lipoprotein cholesterol (HDL-C) levels showed a 55% increase that coincided with a 75% reduction in hepatic lipase activity in postheparin plasma, normalizing after parturition. After delivery, Lp(a) levels showed an almost twofold increase, which could not be explained by the interruption of LDL-apheresis alone, and may be caused by changes in gonadal steroids. Histologic examination of the placenta and the umbilical arteries revealed no atherosclerotic changes, infarctions, or lipid deposits. In general, long-term LDL-apheresis in homozygous FH can delay the onset and complications of severe CAD. In case of a pregnancy, LDL-apheresis seems feasible and should be continued during the pregnancy to prevent superimposed hyperlipidemia and placental insufficiency.

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by a mutation in the gene coding for the low-density lipoprotein (LDL) receptor, and characterized by elevated levels of LDL cholesterol (LDL-C), tendocutaneous xanthomas, and premature coronary artery disease (CAD) [1]. Approximately one in 500 subjects in the population inherits a single copy of the mutant gene of the LDL receptor. These heterozygous patients may develop CAD from the third decade of life. Homozygous patients, usually carrying two different mutant genes for the LDL receptor, are very rare, with a frequency of one in one million persons. In these patients, serum cholesterol levels are extremely high, and they mostly develop CAD in the first or second decades of life. Due to the inability to induce functional LDL receptors, treatment with diet and cholesterol-lowering drugs is never sufficient in these patients, although homozygous patients with some remaining receptor activity can show a certain degree of cholesterol reduction [1,2]. Alternative treatments include portocaval shunting, liver transplantation, and plasmapheresis [3-5]. The latter has been shown to reduce the mortality of CAD [6]. New techniques have been developed to replace plasmapheresis that selectively remove apolipoprotein (apo) B-containing particles extracorporally. These methods make use of immunoabsorption columns, chemical-affinity columns, precipitation with heparin at low pH, and doublefiltration techniques [7-11].

During pregnancy in normal women, the concentrations of all lipids and lipoprotein fractions undergo striking increases due to changes in the levels of estrogens, progestogens, insulin, and human placental lactogen [12,13]. The major lipid increment is in serum triglyceride (TG) levels, usually twofold to threefold, mainly due to an estrogen-induced augmentation of very-low-density lipoprotein synthesis in the liver and probably also by a decrease in lipoprotein lipase activity in the vascular bed and skeletal muscles [14]. Total cholesterol (TC) levels increase up to 60% above nonpregnant levels, an effect that can be ameliorated by a low-cholesterol diet [15]. The LDL-C level increases steadily to term (45 to 50%) and is the only fraction to remain elevated in the puerperium. High- density lipoprotein cholesterol (HDL-C) concentration reaches its maximum at midgestation (+45%) and shows a subsequent decline thereafter.

Pregnancy in homozygous FH women is extremely rare because of the morbidity and mortality of the disease. A few cases have been reported in the literature [16-19]. The main point of concern has been the aggravation of coronary insufficiency of the mother, mainly due to the hemodynamic changes in pregnancy [20,21]. High lipid levels may also affect placental vasculature and cause fetal growth retardation [17,18].

We report the case of an uncomplicated term pregnancy of a 20-year-old woman in whom the diagnosis homozygous FH and severe CAD was established [22]. She

continued treatment with continuous LDL-apheresis using dextran-sulfate cellulose columns throughout her pregnancy [9]. We monitored signs of maternal and fetal distress closely and evaluated lipid and lipoprotein levels.

Subject and methods

Patient

The patient, a young woman of Turkish descent, presented at age 14 with multiple xanthomas, serum TC levels ranging from 15 to 20 mmol/L, and normal serum TG during dietary therapy, and use of resins. After referral to our hospital 1 year later, the diagnosis homozygous FH was made by the presence of hypercholesterolemia in both parents and all six siblings (Table 1), a history of premature myocardial infarction in both parents, fibroblastbinding studies showing a low specific association of LDL of 30%, and a decreased fractional catabolic rate of LDL-apo B of 0.213 pools/d in an LDL turnover study [22]. Family investigation was in accordance with the diagnosis homozygous FH in her youngest sister and heterozygous FH in all the other subjects [22]. The patient had developed angina pectoris during exercise some months before referral. Coronary angiography was performed, which revealed a two-vessel disease among which a 50 to 70% main-stem stenosis of the left coronary artery, making

Table 1. Serum lipid levels (mmol/l) of the proband and the kindred.

	Sex/Age* (yr)	TC	TG	HDL-C	LDL-C#
I-1	M/49	9.56	6.00	0.44	6.82
I-2	F/43	7.22	1.30	0.90	5.98
II-1	M/24	9.54	1.86	1.02	7.85
II-2	F/21	7.45	0.94	1.19	6.05
II-3	F/16	7.38	1.44	1.28	5.68
II-4¶	F/15	16.93	3.86	0.54	14.39
II-5	F/12	7.43	1.50	1.13	5.75
II-6	F/6	16.40	1.12	0.89	15.24
Normal adult		<6.5	<2.0	1.0-1.4	<4.5

**Age at the moment the family has been investigated.*
#Density 1.006-1.063 g/mL
¶Proband

coronary artery bypass grafting necessary. Since then, at the age of 16, biweekly LDL-apheresis (dextran sulfate cellulose columns, MA-01 unit, Kanegafuchi Chemical Industry, Japan) was begun and continued until now, 4 years later. Concomitant treatment with acetylsalicylic acid 160 mg/d and simvastatin was instituted. The latter drug, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor [23], was administered in an attempt to limit the rebound in serum cholesterol after LDL-apheresis, and was stopped 4 months before conception. Anginal attacks did not recur. Planar and tuberoeruptive skin xanthomas on the elbows disappeared within 6 months after the start of LDL-apheresis. At the age of 18, a repeated coronary angiogram revealed no progression of the atherosclerotic lesions [22]. On physical examination at the beginning of the pregnancy, a 20-year-old woman (length 1.55 m, weight 68 kg) with normal blood pressure (135/55 mm Hg) was seen, with inferior corneal lipoid arcs and marked thickening of the Achilles tendons. The electrocardiogram was unremarkable. The patient's spouse had serum lipid levels as follows: TC 6.2 mmol/L, TG 0.89 mmol/L, HDL-C 1.13 mmol/L and LDL-C 4.70 mmol/L.

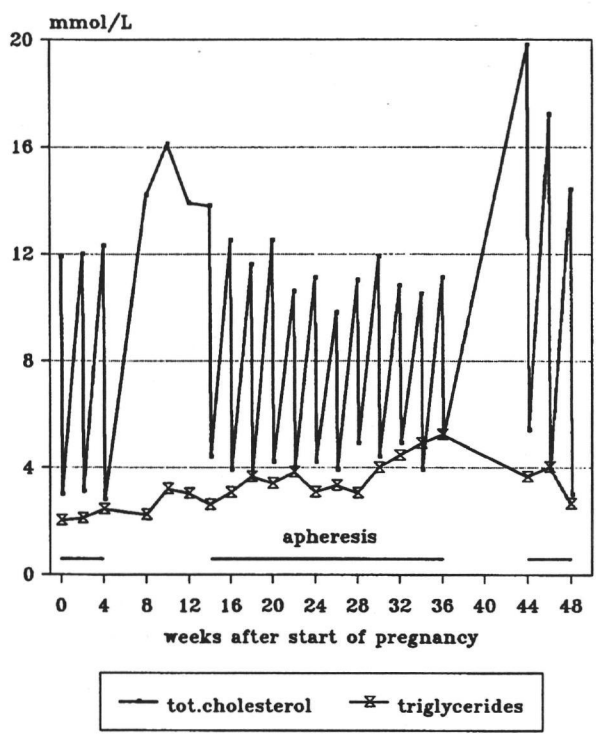

Fig. 1. Concentrations of serum TC and TG (mmol/L) during the course of pregnancy of a patient with homozygous FH, treated with LLDL-apheresissis. Shown are TC levels before and immediately after LDL apheresis and pretreatment TG levels. LDL-apheresis was not performed from 6 to 14 weeks' gestation and for 6 weeks after parturition, from 38 to 44 weeks' amenorrhea

Study protocol

During pregnancy, vital signs were monitored before each treatment with LDL-apheresis, and an electrocardiogram at rest was performed each month. Lipid and lipoprotein levels were determined as described below. Laboratory safety parameters were measured each month, and postheparin lipase activities were measured every 2 months. Fetal growth was determined serially by ultrasonographic measurements of the biparietal diameter, femur length, and abdominal circumference. Starting at 22 weeks' gestation, ultrasonography of the umbilical artery was performed to measure blood flow velocity waveforms (FVWs), both before and after LDL-apheresis using two flow indices, the systolic to diastolic ratio (S/D) and the resistance index ([RI]=(SD)/S), to monitor placental function [24,25]. The maternal placental circulation was also studied by blood FVWs of the left and right uterine artery [25,26]. After parturition, both macroscopic and microscopic aspects of the placenta and umbilical cord were examined. Gestational age was calculated as the number of weeks from the last menstrual period.

Lipid and lipoprotein determinations

After an overnight fast, blood samples were drawn for measurement of serum lipids and lipoproteins, apo A1 and apo B, and lipoprotein(a) [Lp(a)] every 2 weeks. Serum TC and TG were determined by enzymatic methods (CHOD-PAP, no. 237574, Boehringer, Mannheim, Germany, and Sera-PAK, no. 6639, Miles, Italy, respectively). HDL-C was determined using the polyethylene glycol 6000 precipitation method [27]. LDL-C was calculated by subtraction. Samples for apo A1, apo B, Lp(a) and postheparin lipase activities were initially stored at -80°C and determined after the pregnancy. Apo A1 and apo B were quantified in serum by immunonephelometry [28]. Lp(a) was measured by a specific radioimmunoassay (apolipoprotein(a) RIA 100, Pharmacia Diagnostics, Uppsala, Sweden), and lipoprotein lipase and hepatic lipase activities were determined with an immunochemical technique in plasma samples taken 15 minutes after injection of 50 U heparin/kg body weight [29].

The selective removal of apo B-containing lipoproteins with LDL-apheresis causes sawtooth-like alterations in lipoprotein concentrations (Fig 1) [22]. The increase of lipoprotein levels after the treatment is more rapid in the first than in the second week and can be explained by first-order kinetics [5,30]. TC, LDL-C, apo B, and Lp(a) all reach pretreatment levels within 7 to 10 days after apheresis, and have almost identical recovery half-times. Time-averaged concentrations (C_{avg}) of TC, LDL-C, apo B, and Lp(a) can be estimated by the following formula: $C_{avg} = C_{min} + 0.73(C_{max} - C_{min})$, where C_{max} is the pretreatment level and C_{min} the level immediately after the apheresis [31]. We used this formula for the calculation of time-averaged levels of serum TC, LDL-C, apo B, and Lp(a). Serum TG reach the pretreatment levels within 1 to 2 days after apheresis, and HDL-C is not influenced by LDL-

apheresis. Therefore, pretreatment concentrations (C_{max}) of triglycerides and HDL-C were given.

Statistical analysis

Statistical analyses were performed with procedures available in the personal computer software package "Statistix" (NH Analytical Software, St Paul, MN), using the Wilcoxon signed-rank test for paired data and a one-way ANOVA for additional trend analyses. A *P*-value of less than 0.05 was considered significant. All results are expressed as the mean ± SD unless indicated otherwise.

Results

Clinical course

LDL-apheresis was not performed from 6 till 14 weeks' gestation because of the patient's refusal, but was resumed thereafter. The procedures were tolerated well and no adverse effects were apparent. The mean plasma volume treated was 4,583 mL (n=12). The cholesterol-lowering diet was not changed and controlled by a dietician. The patient's body weight increased 11.3 kg during the pregnancy. At 37 weeks' gestation, hospitalization was required because of moderate hypertension and edema of the hands and feet, which subsided within 1 day of bed rest. No signs of cardiac failure or proteinuria were present. Ultrasonography revealed no signs of fetal distress or growth retardation. At 38 weeks' gestation, after spontaneous rupture of the fetal membranes, labor was induced by intravenous synthetic oxytocin. An epidural catheter was inserted and injected with bupivacaine and fentanyl, resulting in an adequate sensory block. Twelve hours after the start of the oxytocin infusion, she delivered a healthy female infant of 3,300 g. Angina pectoris did not occur during labor or in the postpartum period. The infant's 1- and 5-minutes Apgar scores were 9 and 10, respectively. Both mother and infant were discharged from the hospital on the third day postpartum and have subsequently done well. At birth, the neonate's cord blood TC was 1.1 mmol/L and at 2 months of age, while being breast-fed, the serum TC and LDL-C were 5.5 and 4.05 mmol/L, respectively (>95th percentile for controls), confirming obligate heterozygosity for FH [32].

Lipids and lipoproteins

Before and during pregnancy, LDL-apheresis was performed once every 2 weeks. Pretreatment and posttreatment levels of serum TC are presented in Fig 1, showing the typical sawtooth-like changes in concentration. For purposes of clarity, in Fig 2 only the time-averaged levels of LDL-C, Lp(a), and apo B are shown. The concentrations of TC and LDL-C followed the course of treatment with LDL-apheresis (Figs 1 and 2, respectively). LDL-apheresis was not performed from 6 to 14 weeks' gestation

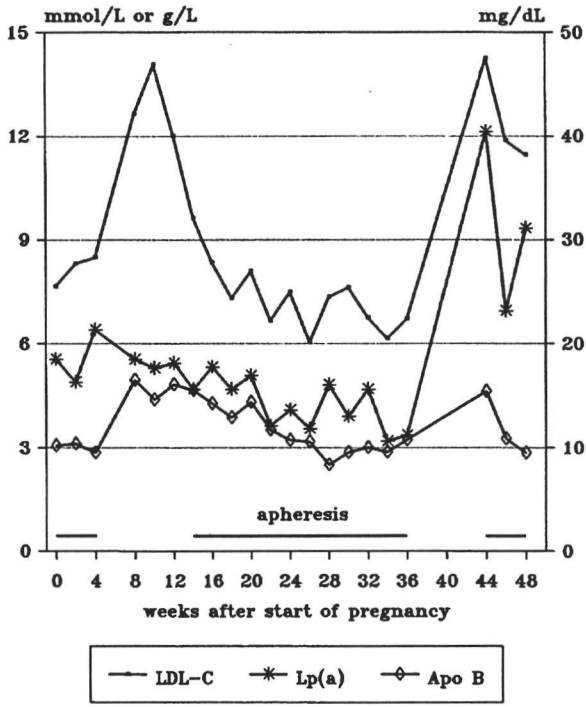

Fig. 2. Time-averaged concentrations of LDL-C (mmol/L), Lp(a) (mg/dL), and apo B (g/L), during the course of pregnancy of a patient with homozygous FH, treated with LDL-apheresis. LDL-apheresis was not performed from 6 to 14 weeks' gestation and for 6 weeks after parturition, from 38 to 44 weeks' amenorrhea

and for 6 weeks immediately following parturition. In these periods, both TC and LDL-C showed a precipitous increase. After resumption of LDL-apheresis during the pregnancy, TC levels immediately before apheresis (C_{max}) were lower than pretreatment levels before the pregnancy (11.22 ± 0.84 *v* 12.76 ± 0.73 mmol/L, $P < .05$), respectively). Posttreatment TC levels (C_{min}) were higher in comparison to treatments before the pregnancy (4.27 ± 0.49 *v* 3.32 ± 0.63 mmol/L, $P < .05$), respectively), although the same plasma volume was treated biweekly (Fig 1). Time-averaged levels of TC were not significantly different in comparison to those in the period before pregnancy (Table 2). Time-averaged levels of LDL-C showed a gradual decline during the pregnancy, and were significantly lower in the third trimester as compared with the prepregnancy levels (Table 2). Apo B levels followed the course of LDL-C during the treatment with LDL-apheresis, showing an increase both in the first trimester and after delivery, the periods in which no apheresis was performed (Fig 2).

Table 2. Mean (±SD) time-averaged concentrations of serum TC, LDL-C, Apo B, and Lp(a) and pretreatment levels of serum TG, HDL-C, and Apo A1 during the course of pregnancy of a patient with homozygous FH, treated biweekly with LDL-apheresis

	TC (mmol/L)	TG (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	Apo A1 (g/L)	Apo B (g/L)	Lp(a) (mg/dL)
pre-pregnancy (n=4)	9.88±0.24	2.21±0.18	8.21±0.38	0.66±0.05	1.52±0.24	3.01±0.29	18.5±2.8
1st trimester (n=5)	12.78±2.80	2.61±0.49	11.10±2.58	0.65±0.05	1.42±0.18	4.03±0.98	18.4±1.8
2nd trimester (n=7)	9.78±1.04	3.29±0.40*	7.64±1.18	1.02±0.11*	1.92±0.23*	3.81±0.62	14.7±2.3¶
3rd trimester (n=5)	9.46±0.43	4.33±0.45*	6.91±0.58*	1.02±0.03*	1.88±0.08*	2.89±0.26#	13.3±2.5¶
post-pregnancy (n=4)	13.17±2.37	3.03±0.99	11.64±2.18	0.68±0.03	1.04±0.09	3.51±1.01	30.8±7.2*

NOTE. LDL-apheresis was not performed during most of the first trimester and 6 weeks after parturition.
*Differences: *P<0.05 v prepregnancy (Wilcoxon signed-rank test), #P<0.05 v 1st and 2nd trimester (ANOVA), ¶P<0.001 v prepregnancy and 1st trimester (ANOVA)*

In the third trimester, apo B concentrations were significantly lower in comparison to the levels in the first and second trimester (Table 2). Trend analysis of time-averaged levels of Lp(a) showed a significant decline of 25% to the end of the pregnancy (Fig 2 and Table 2). In the period during pregnancy in which no apheresis was performed, no increases of Lp(a) concentrations were found, but after parturition, during breastfeeding, Lp(a) showed the same precipitous increase as was found for LDL-C.

Notwithstanding LDL-apheresis, there was a gradual increase of serum TG levels in the third trimester to twice the concentration present before pregnancy (Table 2). After delivery TG levels declined to normal within 2 months (Fig 1). In the second trimester, HDL-C showed a 55% increase to a plateau level, remaining constant in the last trimester (Fig 3). After parturition, HDL-C concentrations returned to prepregnancy levels (Table 2). Apo A1 showed the same change in concentrations as HDL-C (Fig 3 and Table 2). The results of postheparin plasma lipolytic activities are shown in Table 3. At 10 weeks' gestation, lipoprotein lipase activity was within the range for normal female controls [29], showed a decline thereafter, and was still on that lower level 10 weeks after delivery, during breastfeeding. Hepatic lipase activity showed a 75% reduction in the second and third trimester as compared with normal controls, and returned to normal levels after parturition (Fig 3).

Ultrasonic measurements

Fetal growth appeared normal for the biparietal diameter, femur length, and abdominal circumference and ran along the 50th percentile during pregnancy. There were no

Table 3. Postheparin plasma lipoprotein lipase and hepatic lipase activities during the course of pregnancy of a patient with homozygous FH, compared with normals (mean±SD)

Weeks' gestation	Lipoprotein lipase	Hepatic lipase
week 10	7.7	7.4
week 16	4.6	4.7
week 28	3.9	3.7
week 34	3.0	3.8
post-pregnancy	3.7	19.4
normal females (n=15) (29)	8.9±2.2	16.2±6.4

Results are μmol FFA/mL/h

signs of increased placental resistance as measured by the blood FVW of the umbilical artery. Both the S/D and the RI showed a normal gradual decline with the progression of gestation from 2.8 to 1.7 (normal values, 3.9±1.1 to 2.2±0.3) and 0.64 to 0.41 (normal values, 0.75±0.06 to 0.52±0.05), respectively (Table 4) [33]. No differences were found between the RI in the pre- and post-LDL-apheresis state (data not shown). The uterine artery blood FWV indices, only measured in the last trimester, were also within the normal range (data not shown) [26].

Placental examination

The placenta weighed 540 g. Histologic examination of the placenta and umbilical arteries after delivery revealed no atherosclerotic changes, infarctions, or lipid deposits.

Discussion

Pregnancy and delivery in patients with homozygous FH are rare. Only four cases have been reported in the literature (Table 5). Pregnancy in homozygous FH patients treated with long-term LDL-apheresis using dextran-sulfate cellulose columns has not been reported yet. We describe the first pregnancy of a woman with homozygous FH and severe CAD who started treatment with LDL-apheresis 4 years before she became pregnant.

Clinically, we were concerned about the recurrence of angina pectoris and the development of fetal growth retardation. Indeed, there are increased stresses on the cardio-

Table 4. FVWs of the umbilical artery in the preapheresis state, quantitated by the S/D and RI during the course of the pregnancy of a patient with homozygous FH, treated with LDL-apheresis

Weeks'gestation	S/D	RI
week 22	2.80	0.64
week 26	2.40	0.58
week 30	2.28	0.56
week 32	2.25	0.55
week 34	2.25	0.55
week 36	1.70	0.41
normal values (33)	3.90±1.05 to 2.20±0.32	0.75±0.06 to 0.52±0.05

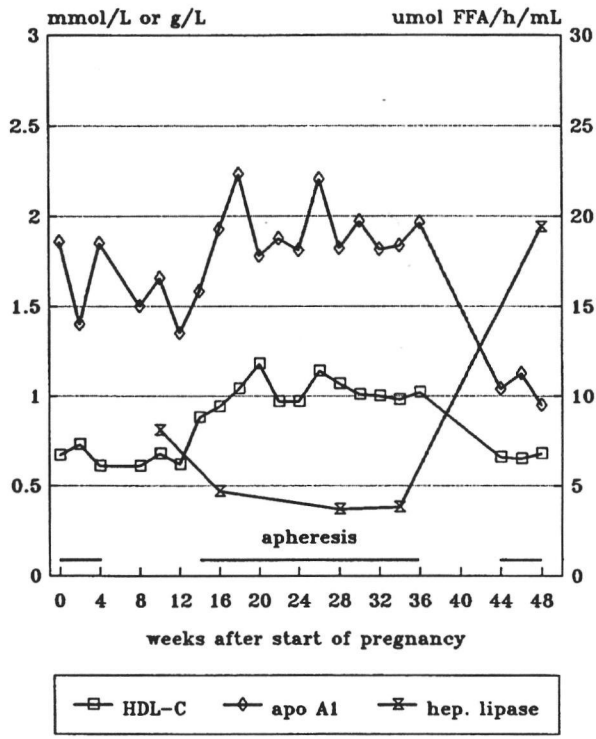

Fig. 3. Pretreatment concentrations of HDL-C (mmol/L) and apo A1 (g/L) and activities of postheparin hepatic lipase (μmol FFA/h/mL) during the course of pregnancy of a patient with homozygous FH, treated with LDL apheresis. LDL apheresis was not performed from 6 to 14 weeks' gestation and for 6 weeks after parturition, from 38 to 44 weeks' amenorrhea

vascular system throughout pregnancy and particularly at delivery. Both blood vol ume and cardiac output increase by 40% to 50%, and during labor cardiac output increases another 25% to 80% [34]. It is clear that these hemodynamic alterations in pregnancy seriously compromise women with ischemic heart disease. In these women, maternal mortality due to myocardial infarction or its complications during pregnancy and within 7 days of delivery is as high as 37% [34]. One case report of a pregnant heterozygous FH patient describes myocardial infarction and death of the mother early in gestation; she had suffered a myocardial infarction 4 years before the pregnancy and had been without complaints until then [20]. The hemodynamic changes in labor are less dramatic during caudal and epidural analgesia, implying that pain is an important component of the cardiovascular stress of labor. Nevertheless, cesarean section also causes serious acute hemodynamic stress [34]. Therefore, vaginal delivery can be accomplished safely during epidural anesthesia [34]. Follow-

up evaluation during pregnancy and postpartum of our patient revealed no increment of coronary insufficiency, and even labor with epidural anesthesia resulted in an uneventful vaginal delivery. Given the course of this pregnancy and delivery, it is likely that long-term LDL-apheresis has prevented further progression of coronary atherosclerosis. Indeed, LDL-apheresis has the potential to prevent progression or even cause regression of CAD in patients with FH [35,36].

Analysis of the uterine and umbilical artery blood FVW has been shown to be a sensitive method for predicting fetal distress during pregnancy and subsequent growth retardation [25]. In normal pregnancy, the arterial blood FVW shows a low-resistance flow pattern, with the S/D and the RI gradually decreasing with advancing gestational age [26,33]. Pathological pregnancy is characterized by increasing resistance in the placental vascular bed, causing a decrease of the end-diastolic flow velocity, and therefore an increment of the S/D and the RI is found [25]. Advanced atherosclerotic changes have not been shown to be the cause of placental insufficiency during the pregnancy of homozygous FH patients (Table 5). Yet, intrauterine growth retardation has been reported in two of four cases [17,18], probably because of hyperviscosity and vasoconstriction in the placental vasculature due to the hyperlipidemic state [18]. Indeed, Beigel et al. reported an increase of the S/D of the umbilical artery during the course of a pregnancy, which became almost normal after plasma exchange [18]. LDL-apheresis has also been shown to change hemodynamics favorably by improving the rheological properties of the blood, and probably also by restoring the endothelium-dependent vasodilation [37,38]. In our case, i.e. continuing biweekly LDL-apheresis during the course of the pregnancy, no signs of fetal growth retardation were recorded, and the studies of the uteroplacental blood FVW did not demonstrate any abnormality. There was a normal decline with the progression of gestation, and even no increase in the pre-LDL-apheresis phase was found, indicating that there were no signs of placental insufficiency due to increased vascular resistance under these metabolic conditions. Additionally, LDL-apheresis was very well tolerated without any maternal or fetal adverse effects. Therefore, these data confirm the observations of Beigel et al. [18] and seem to stress the importance of continuing plasmapheretic treatment in general in homozygous FH patients during pregnancy.

Patients with homozygous FH appear to retain the ability to increase the lipoprotein production in response to the hormonal changes in pregnancy [16]. In our patient, concentrations of TC and LDL-C showed no pregnancy-induced increase, and apo B concentrations paralleled the course of LDL-C levels. Evidently, these changes were due to the biweekly treatment with LDL-apheresis. Serum TG levels instead showed a normal estrogen-induced twofold increase, which is also known in pregnant normocholesterolemic women [12,14]. We did not observe the tremendous 11-fold increase in TG levels that has been described in a homozygous FH case by Tsang et al. [16]. It is not likely that the treatment with LDL-apheresis prevented such an increase, because serum TG returns to pretreatment levels within a few days

Table 5. Characteristics of homozygous FH patients during the course of pregnancy and delivery, as reported in the literature.

Author	Medical history	Change in Lipid Levels (%)				Treatment	Delivery	Cardiac events	Placental insufficiency	Fetal growth retardation
		TC	TG	HDL	LDL					
Tsang, 1978 (16)	MI	+19	+1095	+39	+12	diet	section	ischemia	no (hist)	no
	CABG					cholestyramine	37 weeks	postpart.		
Barss, 1985 (17)	-	+28	-	-	-	diet	induced	no	no (hist)	yes, 2250 g
							38 weeks			
Beigel, 1990 (18)	AP	+147*	-	-	-	diet	normal	no	no (echo)	yes, 2700 g
						plasma exchange	39 weeks			
Goldstein, 1991 (19)	PTCA	-	-	-	-	diet	normal	no	no (hist)	no
						plasma exchange				-
Kroon (present study)	CABG	-9*	+96*	+55*	-17*	diet	normal	no	no (echo+hist)	no, 3300 g
						LDL-apheresis	38 weeks			

*Abbreviations: MI, myocardial infarction; CABG, coronary artery bypass graft; AP, angina pectoris; PTCA, percutaneous transluminal coronary angiography; hist, histological examination of placenta and umbilical cord; echo, ultrasound analysis of blood FVW of the umbilical artery. *concentrations determined immediately before LDL-apheresis have been used for comparison*

after an apheresis [31,39].

Notwithstanding the performance of LDL-apheresis, the gradual decrease of LDL-C and apo B in comparison to prepregnancy levels may be caused by the action of estrogens, which are known to increase LDL degradation through induction of LDL receptors [40,41]. In a case report, Mabuchi et al. described the estrogen-induced normalization of TC and LDL-C, together with the reduction of tendon xanthomas, during pregnancy in a heterozygous FH female due to increased expression of LDL receptors [42]. Our patient was known to have a small percentage of (mutant) LDL receptor activity, and treatment with simvastatin (an hepatic hydroxymethyl-glutaryl-coenzyme A reductase-inhibitor) at a dose of 40 mg/d had been shown to induce a limited decrease of TC of 11% [22]. Therefore, increasing concentrations of estrogens during her pregnancy may have caused the further decrease of LDL-C and apo B levels in the second and third trimester as compared with prepregnancy levels.

In two studies, Lp(a) levels have been monitored during pregnancy [43,44]. The first one reported an increment of Lp(a) levels to a maximum in the middle of the second trimester [43]. In contrast, the second found an increase of Lp(a) until the end of pregnancy, which paralleled the changes well known for plasma TG, TC, and apo B, suggesting modulation by endogenous hormones [44]. Lp(a) levels in our patient did not increase and even showed a 25% decrease in the second and third trimester. These changes are caused by LDL-apheresis, which has been shown to induce an acute decrease of 70% of Lp(a) concentrations and to decrease time-averaged Lp(a) levels by 20% depending on the frequency and volume of plasma treated [31]. The almost twofold increase of Lp(a) levels after delivery in comparison to the period in which no LDL-apheresis was performed in the first trimester is of interest. Hormonal changes after parturition may have caused the increase of Lp(a) levels at that time, since interruption of treatment in the first trimester was not followed by such an increase. This observation also suggests a role for gonadal steroids in the regulation of Lp(a) levels, as has been reported recently [45-47].

The pattern of HDL-C increase - a 55% increment to a plateau in the second and third trimester - was greater than previously reported for normal subjects [12,13] and a homozygous FH case [16], but the subsequent decrease after parturition to preconception levels in the homozygote paralleled that reported for normals. In pregnancy, estrogens have been shown to cause an increase of very-low-density lipoprotein synthesis and a decrease in overall plasma TG removal capacity as measured by postheparin lipolytic activity [14,48]. Although redistributed within certain tissues, lipoprotein lipase activity decreases with the progression of gestation, which occurs when maternal TG levels are highest. Hepatic lipase activity shows an even more pronounced reduction during the course of pregnancy. Both lipoprotein lipase and hepatic lipase contribute significantly to the variation in HDL-C levels in a reciprocal manner [49,50]. In our case, the estrogen-induced decrease of hepatic

lipase activity may have caused the observed increase of HDL-C. This is supported by the simultaneous increase of apo A1, which is known to be increased by high estrogen levels [51].

In summary, the pregnancy and delivery of this patient with homozygous FH and severe CAD, who was treated with LDL-apheresis throughout her pregnancy, did not coincide with maternal and fetal distress or gross deterioration of lipid metabolism. In general, long-term LDL-apheresis seems feasible in pregnant homozygous FH women. This form of apheresis can delay the onset and progression of CAD and may allow the patients to have offspring. Nevertheless, pregnancy and delivery in women with ischemic heart disease is hazardous and should be monitored very closely. LDL-apheresis should be continued during the pregnancy course to control superimposed hyperlipidemia and placental insufficiency and subsequent intrauterine growth retardation.

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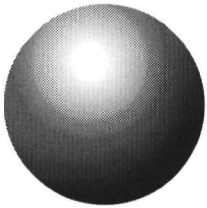


Chapter

Eleven

Summary and conclusions

Summary and conclusions

The main issue of the present thesis was to study the effect of aggressive lowering of low density lipoprotein (LDL) cholesterol on the severity of established cardiovascular disease. The mechanisms by which LDL-cholesterol lowering promotes arrest of progression of atherosclerotic vascular disease includes a combination of reduction of atherogenic LDL-particles, decreased susceptibility of LDL to oxidative modification, reduction of the atherosclerosis-associated inflammatory responses in the vessel wall, improvement of the endothelium-dependent vasodilation, and stabilization of rupture-prone lesions.

More effective lipid lowering has become available with the introduction of HMG-CoA reductase inhibitors, e.g. pravastatin or simvastatin. In chapter 2 we showed that a 51% reduction of plasma total cholesterol by the administration of high doses of pravastatin for 9 months in young Watanabe heritable hyperlipidemic (WHHL) rabbits reduced the incidence of both coronary and aortic atherosclerotic plaques in comparison with untreated animals. Basal coronary flow and bradykinin-induced increase in coronary flow were higher than in the control animals and comparable with normocholesterolemic New Zealand white rabbits. Moreover, the change in bradykinin-induced flow was greater in the animals with lower plasma total cholesterol levels. This indicates that in this model cholesterol lowering with pravastatin not only retards the progression of plaque formation, but also preserves endothelium-dependent relaxation of the coronary arteries.

An alternative approach of cholesterol lowering is LDL-apheresis using dextran sulfate cellulose columns. This treatment selectively removes large amounts of apolipoprotein B- (apo B) containing lipoproteins from plasma, i.e. VLDL and LDL, by chemical binding of apo B to dextrans. We applied biweekly LDL-apheresis in combination with the administration of simvastatin in an open randomized 2-years trial, the LDL-Apheresis Atherosclerosis Regression Study (LAARS), as has been described in the chapters 3 to 7 and 9. The effects of this more aggressive cholesterol lowering treatment in comparison to conventional treatment with simvastatin alone were evaluated in 42 hypercholesterolemic men with extensive coronary artery disease (CAD). Primary outcome measures were changes in CAD, as measured by quantitative coronary angiography and the angiographic assessment of the regional myocardial blood flow, whereas secondary outcome measures were changes in peripheral vascular disease (PVD), as measured noninvasively by ultrasound techniques (chapter 3).

LDL-apheresis causes sawtooth-like alterations in plasma lipoprotein concentrations. The efficacy of the treatment depends on the pre- and posttreatment lipid levels, and on the rate of return to pretreatment levels, the so-called rebound. In chapter 4 the rebound of lipids and lipoproteins from the subjects of LAARS were described. We showed that a single LDL-apheresis removed roughly 5 to 6% of the total exchangeable

cholesterol body stores. Although all patients were treated concomitantly with the cholesterol synthesis inhibitor simvastatin at a dose of 40 mg/day, the pretreatment ratio of lathosterol to cholesterol was increased to 3.60±1.45, indicating that long-term aggressive lipid lowering had increased whole-body cholesterol synthesis. By nonlinear regression analysis of the rebound curves the recovery half-times of LDL and Lp(a) were comparable, 4.0 and 3.5 days, respectively, whereas return to pretreatment levels took 13 and 12 days, respectively. This indicates that the interval of biweekly treatments is acceptable. Additionally, in this chapter a simple uniform formula was presented for the estimation of the mean plasma levels between two apheresis procedures using only pre- and posttreatment levels .

In chapter 5 we described that a single LDL-apheresis improved all parameters for in-vitro copper-induced LDL-oxidizability, lag-time, maximal rate of diene production, and time to maximal diene production, until the 3rd day after apheresis. This could be explained by changes in the fatty acid composition, since the linoleic acid and arachidonic acid content of LDL were reduced by 11 and 18%, respectively. Although large amounts of the main lipophilic antioxidant of LDL, vitamin E, were removed, the vitamin E content per LDL-particle did not change acutely, nor in the days of the recovery period. Since the biological consequences of the rather short time-span of less susceptibility to LDL-oxidation are unknown, it is concluded that LDL-apheresis at least does not affect LDL-oxidizability in a negative way.

In chapter 6 and 7 we described the outcome of long-term aggressive lipid lowering treatment on CAD. Baseline LDL-cholesterol was lowered by an average of 63% (from 7.8±1.9 to 3.0±1.1 mmol/L) and by 47% (from 7.9±2.3 to 4.1±1.6 mmol/L) in the apheresis and medication group, respectively. Quantitative coronary angiography showed no differences between both groups in coronary artery mean segment diameter (MSD), -0.01±0.16 versus 0.03±0.16 mm, respectively, nor in minimal obstruction diameter (MOD), -0.01±0.13 versus 0.01±0.11 mm, respectively. This means that complete arrest of progression of CAD was achieved in both groups after 2 years of treatment. However, bicycle exercise tests in the apheresis group showed a significant prolongation of the ischemic threshold on the ECG, the time to 0.1 mV ST-depression, from 461±47 to 641±50 sec, in comparison with no change (485±81 to 442±60 sec) in the medication group. This may indicate that functional improvements preceded anatomical changes (chapter 6). Indeed, regional myocardial blood flow assessed by digital subtraction angiography with videodensitometric calculation of the hyperemic mean transit time (HMTT) showed a significant improvement from 3.4±1.2 to 2.9±0.8 sec in the apheresis group versus no change (3.0±1.1 to 3.0±0.9 sec) in the medication group (chapter 7). Categorizing patients into groups with increased versus reduced time to 0.1 mV ST-depression showed a 16% decrease of HMTT in the group with an improved versus a 4% increase in HMTT in the group with a worsened ischemic threshold (P=0.04). Therefore, more aggressive lipid lowering increased the ischemic exercise-

induced threshold by improving the myocardial blood flow, i.e. restoring the endothelialdependent and/or independent relaxation in the coronary circulation.

Assessments of peripheral vascular atherosclerosis were performed in the aortotibial tract and in the carotid arteries. In the aortotibial tract a combination of the ankle/arm systolic blood pressure index (AAI) and Doppler spectrum analysis (DOSA) of the femoral arteries, at rest and during reactive hyperemia was used to measure hemodynamically significant pathology. In the carotid arteries early atherosclerotic changes were assessed by B-mode echography of the intima-media thickness (IMT) of the near and far wall of the common carotid artery, the bifurcation, and the internal carotid artery. In chapter 8 we showed that the prevalence of hemodynamically significant PVD in the lower extremities in patients with heterozygous familial hypercholesterolemia (FH) was 31% compared to 4% in age-matched normocholesterolemic control subjects. The first manifestations of PVD were already present at the age of 30. These data indicate that PVD is much more prevalent in FH patients than is generally assumed. We also showed that men and women were equally affected at all ages in contrast to the prevalence of CAD in FH which presents approximately 10 years earlier in men compared to women.

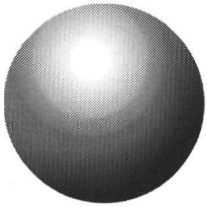
Sequential measurements of PVD in LAARS were performed and described in chapter 9. The number of sites with hemodynamic stenoses in the right and/or left aortotibial tract was reduced from 17 to 13 in the apheresis group and increased from 11 to 23 in the medication group (P=0.002). Categorization of all patients in those who showed an increase in the number of hemodynamic aortotibial stenoses (n=15) versus those who improved or showed no change (n=26) revealed that the latter group had significantly lower LDL-cholesterol, lipoprotein(a) [Lp(a)], and apo B levels. The analyses of the IMT of the carotid arteries showed comparable changes: mean IMT of all carotid segments in the apheresis group regressed from 0.91±0.38 to 0.85±0.29 mm but thickened from 0.88±0.30 to 0.93±0.40 mm in the medication group (P<0.001). Changes in IMT were predominantly found in the common carotid artery and the bifurcation, and were associated with changes in Lp(a) levels (r=0.59, P=0.004). So, both early and hemodynamically significant atherosclerotic lesions regressed by more aggressive lipid lowering. This indicates that although atherosclerosis is a generalized condition, the impact of lipid lowering may be different for the coronary and the peripheral vascular beds.

Finally, in chapter 10 we described a patient with homozygous FH on long-term LDL-apheresis treatments during a pregnancy. She was known with severe CAD from the age of 15 and treated with biweekly LDL-apheresis and simvastatin since then. Mean time-averaged LDL-cholesterol levels during treatment forgoing the pregnancy were 8.2±0.4 mmol/L. During the course of the pregnancy, while apheresis was continued, she developed no signs of coronary insufficiency, neither during delivery. Serial Doppler spectrum measurements of the uterine and umbilical artery did not show signs

of insufficiency of the uteroplacental circulation. It is postulated that long-term LDL-apheresis is the therapy of choice in homozygous FH and is able to prevent atherosclerotic complications, even during pregnancy.

Conclusions that were drawn from these studies:

1. In WHHL rabbits lipid lowering with high doses of pravastatin retards the progression of atherosclerosis and preserves endothelium-dependent vasodilation.
2. In hypercholesterolemic patients (total cholesterol >8.0 mmol/L) with extensive coronary atherosclerosis lipid lowering with simvastatin alone is able to arrest the progression of CAD, but apparently insufficient to improve myocardial blood flow or prevent the progression of PVD.
3. More aggressive lipid lowering with LDL-apheresis and simvastatin in the same population arrests the progression of CAD and also improves myocardial blood flow, and moreover, induces regression of PVD.
4. Functional improvements of endothelial vasomotion precede anatomical changes in the process of regression of atherosclerosis.
5. The impact of lipid lowering is different between coronary and peripheral vascular beds.
6. Biweekly long-term LDL-apheresis is a safe and effective therapy, which should be considered in drug refractory hypercholesterolemic patients with established CAD.
7. During LDL-apheresis time-averaged concentrations of total cholesterol, LDL-cholesterol, Lp(a), and apo B can be uniformly estimated by the following formula: $C_{AVG}=C_{MIN}+0.73(C_{MAX}-C_{MIN})$.
8. A single LDL-apheresis procedure induces short-term, favourable changes in LDL-oxidizability by changes in the composition of the LDL particles.
9. Hemodynamically significant PVD is present in approximately one third of subjects with heterozygous FH without differences in gender.



Chapter

Twelve

Samenvatting en conclusies

Samenvatting en conclusies

Het belangrijkste onderwerp van dit proefschrift was het bestuderen van het effect van rigoreuze verlaging van het lage dichtheidslipoproteïne (low density lipoprotein, LDL, dat grotendeels uit cholesterol bestaat), op de progressie van reeds bestaand hart- en vaatlijden. De mechanismen die leiden tot een afname van de voortgang van atherosclerotisch vaatlijden ('aderverkalking') zijn diverse. Te weten: afname van de concentratie van de atherogene LDL-deeltjes, verminderde gevoeligheid van LDL voor oxidatieve verandering, vermindering van de met atherosclerose samenhangende ontstekingsprocessen in de vaatwand, verbetering van endotheel-afhankelijke bloedvatverwijding, en het stabiliseren van atherosclerotische lesies van de bloedvatwand die makkelijk kunnen ruptureren.

Effectievere verlaging van het cholesterolgehalte in het bloed is beschikbaar geworden met de introductie van een groep geneesmiddelen die de aanmaak van cholesterol remmen, HMG-CoA reductase remmers genoemd, zoals bijvoorbeeld pravastatine of simvastatine. In hoofdstuk 2 staat een studie beschreven met jonge Watanabe konijnen, een speciale stam met een erfelijke aandoening waardoor het cholesterolgehalte in het bloed sterk verhoogd is. Een deel van deze konijnen werd gedurende 9 maanden behandeld met hoge doses pravastatine. In die studie laten we zien dat een vermindering van 51% van het totale cholesterolgehalte in het bloed zowel het optreden van atherosclerotische afwijkingen in de bloedvaten van het hart (de coronairarterieën) als in de lichaamsslagader (aorta) verminderd heeft in de behandelde dieren. Ook de basale bloeddoorstroming in het hart en de door bradykinine geïnduceerde toename van de bloeddoorstroming in het hart waren hoger in de behandelde konijnen in vergelijking met de controle groep. Deze waarden waren vergelijkbaar met die van konijnen met een normaal cholesterolgehalte. Bovendien was de verandering die teweeg gebracht werd door bradykinine groter in die dieren die een lager cholesterolgehalte in het bloed hadden. Dit betekent dat, in deze proefopstelling, het verlagen van cholesterol met pravastatine niet alleen de verdere progressie van het vormen van atherosclerotische afwijkingen tegengaat, maar dat ook de endotheel-afhankelijke verwijding van de bloedvaten van het hart behouden blijft.

Een alternatieve manier om het cholesterolgehalte te verlagen is LDL-aferese met behulp van dextraansulfaat cellulose kolommen ('vetspoeling van het bloed'). Deze behandeling verwijdert selectief grote hoeveelheden lipoproteïnen uit het bloed die het eiwit apolipoproteïne B (apo B) bevatten, namelijk VLDL en LDL. Dit gebeurt door chemische binding van apo B aan dextranen. In een open, gerandomiseerde, 2 jaar durende studie, de LDL-Apheresis Atherosclerosis Regression Study (LAARS) behandelden wij patiënten met LDL-aferese, 1x per 2 weken, in combinatie met het medicament simvastatine. Dit staat beschreven in de hoofdstukken 3 t/m 7 en 9. De effecten van deze meer agressieve manier om het cholesterolgehalte te verlagen werden vergele-

ken met de gebruikelijke behandeling met alleen simvastatine. Het onderzoek werd uitgevoerd bij 42 mannen met een sterk verhoogd cholesterolgehalte en met uitgebreid vaatlijden van het hart (coronary artery disease, CAD). De effecten van de behandeling werden gemeten met kwantitatieve coronaire angiografie en met bepaling van de regionale bloeddoorstroming in de hartspier gemeten met digitale subtractie technieken. Tevens werden de veranderingen in perifere bloedvaten (in hals en benen, peripheral vascular disease, PVD) gemeten met echografische methoden (hoofdstuk 3).

LDL-aferese veroorzaakt zaagtandachtige veranderingen in de concentraties van lipoproteïnes. De effectiviteit van de behandeling hangt af van de bloedwaardes vóór en na de behandeling en van de snelheid waarmee de concentraties van vóór de behandeling weer bereikt worden, de zogenaamde rebound. In hoofdstuk 4 wordt de rebound bij de patiënten uit LAARS beschreven. We toonden aan dat door een eenmalige LDL-aferese ongeveer 5 tot 6% van het uitwisselbare cholesterol in het lichaam, daadwerkelijk verwijderd wordt. Hoewel alle patiënten tegelijkertijd behandeld werden met de cholesterol synthese remmer simvastatine in een dosis van 40mg/dag, was de ratio van lathosterol/cholesterol vóór de aferese verhoogd tot 3.60±1.45. Dit geeft aan dat langdurige agressieve cholesterolverlaging de totale synthese van cholesterol in het lichaam doet toenemen. Niet lineaire regressieanalyse van de rebound grafieken van LDL en Lp(a) liet zien dat de halveringstijd voor de toename van de concentratie na aferese vergelijkbaar was (respectievelijk 4.0 en 3.5 dagen). Het duurde respectievelijk 13 en 12 dagen voordat de waarden van vóór de behandeling weer bereikt waren. Dit geeft aan dat de tweewekelijkse intervallen voor de aferese acceptabel zijn. Daarnaast is in dit hoofdstuk een eenvoudige formule beschreven waarmee uit de concentratie vóór en direct na de behandeling de gemiddelde concentratie tussen twee afereses bepaald kan worden.

In hoofdstuk 5 beschrijven we dat een eenmalige LDL-aferese alle parameters verbeterd van de door koper geïnduceerde oxideerbaarheid van LDL. Dit bleef aanwezig tot de derde dag na de aferese. Het kon verklaard worden door veranderingen in de vetzuur samenstelling van LDL, omdat de hoeveelheid linoleenzuur en arachidonzuur verminderd waren met respectievelijk 11 en 18%. Hoewel grote hoeveelheden van het lipofiele antioxidant van LDL, vitamine E, verwijderd waren, veranderde het gehalte van vitamine E per LDL-deeltje niet. Omdat de biologische gevolgen van de vrij korte tijd van verminderde gevoeligheid voor oxidatie van LDL niet bekend is, concludeerden we dat LDL-aferese de LDL-oxideerbaarheid in ieder geval niet negatief beïnvloedt.

In hoofdstuk 6 en 7 worden de resultaten van LAARS beschreven ten aanzien van het coronairlijden. Het LDL-cholesterol in de aferesegroep was gemiddeld verminderd met 63% (van 7.8±1.9 tot 3.0±1.1 mmol/L) en in de medicatiegroep met 47% (van 7.9±2.3 tot 4.1±1.6 mmol/L). De kwantitatieve coronaire angiografie liet geen verschillen tussen de twee groepen zien in de gemiddelde segment diameter van de coronair

arterieën (MSD), -0.01±0.16 in vergelijking met 0.03±0.16 mm. En ook niet in de gemiddelde diameter van de ergste stenose (MOD), -0.01±0.13 in vergelijking met 0.01±0.11 mm. Dit betekent dat volledige stilstand van de progressie van het coronair vaatlijden was bereikt in beide groepen na 2 jaar behandeling. Echter, bij de fietstesten bleek in de aferesegroep een significante verlenging te zijn opgetreden van de tijd voordat zuurstoftekort in de hartspier ontstond. Dit werd gemeten op het ECG als de tijd totdat een daling optrad van 0.1mV in het ST-segment. In de aferesegroep nam deze tijd toe van 461±47 tot 641±50 sec, terwijl er in de medicatiegroep geen verandering werd gezien (485±81 tot 442±60 sec). Dit zou er op kunnen wijzen dat verbeteringen in de functie van de bloedvaten vooraf gaan aan anatomische veranderingen (hoofdstuk 6). Inderdaad bleek de regionale bloeddoorstroming van de hartspier, berekend aan de hand van de gemiddelde passagetijd van het contrast tijdens maximale vaatverwijding (HMTT), significant verbeterd te zijn in de aferese groep (van 3.4±1.2 tot 2.9±0.8 sec) en niet veranderd in de medicatiegroep (van 3.0±1.1 tot 3.0±0.9 sec). Als de patiënten ingedeeld werden in een groep die een verbetering liet zien bij de fietstest en in een groep die geen verbetering liet zien, dan werd in de verbeterde groep 16% reductie van de HMTT en in de niet verbeterde groep een toename van 4% van de HMTT gevonden (P=0.04). Dus het agressiever verlagen van het cholesterolgehalte verminderde het zuurstoftekort van het hart bij inspanning door de bloeddoorstroming van de hartspier te verbeteren. Dit wordt waarschijnlijk verklaard door het verbeterde vermogen van de bloedvaten om zich te verwijden onder invloed van endotheel-afhankelijke en -onafhankelijke factoren (hoofdstuk 7).

Metingen van atherosclerose in de perifere vaten werd gedaan in de grote slagaders naar de benen (aortotibiale gebied) en in de slagaders in de hals (arteria carotis). Voor de benen werd een combinatie gebruikt van de ratio van de systolische bloeddruk in de enkel en de arm (enkel/arm druk) en van Doppler spectrum analyse (DOSA) van de bloedvaten in de bovenbenen (arteria femoralis). Dit werd gemeten zowel in rust als tijdens maximale vaatverwijding (hyperemie) en is een maat voor vernauwingen die de bloeddoorstroming belemmeren (hemodynamisch belangrijke stenosen). In de halsvaten werden juist vroege atherosclerotische afwijkingen gemeten door middel van echografische bepaling van de intima-media dikte (IMT) van de vaatwand. Dit werd uitgevoerd op 3 plaatsen in de halsslagader. Gebruik makend van de enkel/arm druk en de DOSA van de arteria femoralis laten we in hoofdstuk 8 zien dat bij patiënten met een heterozygote vorm van familiaire hypercholesterolemie (FH) hemodynamisch belangrijke stenosen in de bloedvaten naar de benen veel frequenter voorkomen (31%) dan bij een groep van gelijke leeftijd en met een normaal cholesterolgehalte (4%). De eerste verschijnselen van perifeer vaatlijden bij deze groep FH-patiënten waren reeds aanwezig op de leeftijd van 30 jaar. Deze gegevens laten zien dat perifeer vaatlijden veel vaker voorkomt bij FH-patiënten dan algemeen wordt aangenomen. We toonden ook aan dat er bij deze patiënten geen verschil is tussen mannen en vrouwen in de

leeftijd waarop zij zich presenteren met de eerste verschijnselen van perifeer vaatlijden in tegenstelling tot vaatlijden aan het hart dat bij mannen ongeveer 10 jaar eerder begint.

De diverse metingen van het perifere vaatlijden bij LAARS zijn beschreven in hoofdstuk 9. Het aantal hemodynamisch belangrijke vernauwingen in de vaten naar de benen nam af van 17 tot 13 in de aferese groep en nam toe van 11 tot 23 in de medicatiegroep (P=0.002). Als alle patiënten werden verdeeld in een groep die een toename in het aantal vernauwingen liet zien (n=15) in vergelijking met de patiënten die geen verandering of een afname lieten zien, konden we vaststellen dat de onveranderde/verbeterde groep een significant lager LDL-cholesterol, lipoproteïne(a) [Lp(a)] en apo B gehalte had. De analyses van de IMT van de halsslagaders liet vergelijkbare veranderingen zien: de gemiddelde IMT van alle segmenten van de halsslagaders verminderde in de aferesegroep van 0.91±0.38 tot 0.85±0.29 mm maar werd dikker in de medicatiegroep (van 0.88±0.30 tot 0.93±0.40 mm, P<0.001). Veranderingen in IMT waren geassocieerd met veranderingen in het Lp(a) gehalte (r=0.59, P=0.004). Dus zowel hemodynamisch significante atherosclerotische stenosen als vroege afwijkingen namen af tijdens agressieve cholesterolverlaging maar niet onder de gebruikelijke therapie. Hoewel atherosclerose een gegeneraliseerde ziekte is, is het effect van cholesterolverlaging verschillend voor de perifere bloedvaten en voor de bloedvaten van het hart.

Tenslotte hebben we in hoofdstuk 10 een patiënte beschreven met een homozygote FH behandeld met langdurige LDL-aferese tijdens de zwangerschap. Vanaf haar 15de jaar was zij bekend met ernstig vaatlijden van het hart en wordt sindsdien behandeld met tweewekelijks LDL-aferese en simvastatine. De gemiddelde LDL-cholesterol gehaltes onder behandeling voorafgaande aan de zwangerschap waren 8.2±0.4 mmol/L. Tijdens de zwangerschap, terwijl de LDL-aferese werd voortgezet, ontstonden er geen angineuze klachten. Dit gebeurde ook niet tijdens de bevalling. Herhaalde Doppler spectrum metingen van de vaten van de baarmoeder en de navelstreng gedurende de zwangerschap lieten geen tekenen zien van onvoldoende doorbloeding van de uterus en placenta. Deze casus toont aan dat langdurige LDL-aferese de behandeling van keuze is bij patiënten met homozygote FH en dat hiermee belangrijke cardiovasculaire complicaties werden voorkomen, zodat deze patiënte zelfs een zwangerschap kon voldragen.

Conclusies die uit deze onderzoeken getrokken kunnen worden:

1. Verlaging van het cholesterolgehalte bij jonge Watanabe konijnen met hoge doses pravastatine remt de progressie van atherosclerose en houdt de endotheel-afhankelijke verwijding van de bloedvaten in stand.
2. Cholesterolverlaging met simvastatine alleen bij patiënten met een sterk verhoogd cholesterolgehalte (>8.0 mmol/l) en uitgebreid coronarialijden remt de progressie

van coronarialijden maar verbetert niet de doorbloeding van de hartspier noch voorkomt het progressie van het perifere vaatlijden.

3. De meer agressieve wijze van het verlagen van het cholesterolgehalte met LDL-aferese en simvastatine in eenzelfde populatie remt de progressie van coronarialijden, verbetert de doorbloeding van de hartspier en veroorzaakt regressie van het perifere vaatlijden.
4. Tijdens het proces van regressie van atherosclerose lopen functionele veranderingen vooruit op de anatomische.
5. De verlaging van het cholesterolgehalte heeft verschillende effecten op de vaten van het hart en de perifere vaten.
6. Langdurige behandeling met LDL-aferese is een veilige en effectieve therapie. Deze behandeling moet overwegen worden bij patiënten met coronarialijden bij wie het cholesterolgehalte niet adequaat kan worden verlaagd met medicatie alleen.
7. Tijdens LDL-aferese kunnen gemiddelde concentraties over de tijd van het totaal cholesterol, LDL-cholesterol, Lp(a) en apo B allemaal met dezelfde formule geschat worden: $C_{GEM}=C_{MIN}+0.73(C_{MAX}-C_{MIN})$.
8. Een eenmalige LDL-aferese veroorzaakt kortdurige, gunstige veranderingen in de oxideerbaarheid van LDL door veranderingen in de samenstelling van de LDL-deeltjes.
9. Bij één op de drie patiënten met heterozygote FH treft men hemodynamisch significant perifeer vaatlijden aan, onafhankelijk van het geslacht.

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Anton Stalenhoef is de initiator en drijvende kracht achter LAARS geweest. Beste Anton, jij bent een gedreven onderzoeker, en ik heb veel van je geleerd. Het onderzoek heeft mede de basis gelegd voor mijn huidige functie. Prof. dr. Ab van 't Laar was altijd op de achtergrond aanwezig. Beste Ab, door jouw emeritaat zijn onze discussies over mijn onderzoek enigszins achterwege gebleven. De jaren dat ik bij jou in opleiding was, waren heel plezierig. Jij bent voor mij als arts een groot voorbeeld. Uiteraard gaat mijn dank ook uit naar Paul Stuyt, Marc Mol en Bas Bredie van wie en met wie ik de lipidologie in de vingers kreeg. Marc, we waren een goed team.

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Verder wil ik iedereen bedanken die de afgelopen jaren voor Féline en Maxime hebben gezorgd, zodat aan het proefschrift gewerkt kon worden. Hierbij wil ik met name mijn schoonouders noemen als echte rotsen in de branding.

Lieve Désirée, dank je wel voor al je steun, onzichtbaar en zichtbaar in de layout van dit boekje. Je bent een reus die veel meer kan dragen dan een instabiel bekken doet vermoeden. Beiden full-time werken en veel reizen maakt het er zeker niet rustiger op, maar nu lijkt toch echt de periode aan te breken dat we meer tijd voor elkaar en de kinderen krijgen.

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Curriculum Vitae

Bram Kroon werd op 3 april 1959 geboren te Geleen. Na het behalen van het diploma Atheneum-B aan de Albert Schweitzer Scholengemeenschap te Geleen, startte hij in 1977 met de studie geneeskunde aan de Medische Faculteit van de Katholieke Universiteit te Nijmegen. Het doctoraalexamen werd behaald in 1982 en het artsexamen in juni 1984. Vanaf augustus 1984 was hij werkzaam op de afdeling Algemene Interne Geneeskunde in het St. Radboudziekenhuis te Nijmegen (hoofd: prof.dr. A. van 't Laar). In januari 1985 startte hij met zijn opleiding tot internist in het St. Maartens Gasthuis te Venlo (opleider: dr. J.J.J. Mattousch) en vervolgde deze vanaf januari 1987 in het St. Radboudziekenhuis te Nijmegen (opleider: prof. dr. A. van 't Laar). Op 1 januari 1990 werd hij door de Specialisten Registratie Commissie ingeschreven als internist en was werkzaam op de afdeling Algemene Interne Geneeskunde (hoofd: prof. dr. J.W.M. van der Meer). Vanaf augustus 1989 tot mei 1995 verrichtte hij daar het in dit proefschrift beschreven onderzoek onder leiding van dr. A.F.H. Stalenhoef. Sedert mei 1995 is hij als algemeen internist met speciale belangstelling voor hypertensie en hyperlipoproteïnemieën verbonden aan de afdeling Interne Geneeskunde van het Academisch Ziekenhuis te Maastricht (hoofd: Prof. dr. H.F.P. Hillen).
Hij is getrouwd met Désirée van der Heijde. Zij hebben twee dochters, Féline (1992) en Maxime (1994).

Stellingen

behorende bij het proefschrift

'Aggressive cholesterol lowering and regression of atherosclerosis

Studies on LDL-apheresis'

Bram Kroon, 9 oktober 1996

1. Bij secundaire preventie van hart- en vaatziekten geldt t.a.v het serumcholesterolgehalte: hoe lager hoe beter.
   Dit proefschrift

2. Voorafgaande aan de angiografische afname van atherosclerose treedt functioneel herstel op van de reactiviteit van de vaatwand.
   Dit proefschrift

3. Aan het arsenaal van uitkomstvariabelen van toekomstige regressiestudies dienen functionele testen te worden toegevoegd.
   Dit proefschrift

4. Het gebruik van een HMG-CoA reductase remmer voorkomt de toename van de cholesterolsynthese tijdens behandeling met LDL-aferese niet.
   Dit proefschrift

5. LDL-aferese moet alleen overwogen worden als medicamenteus het serumcholesterolgehalte niet voldoende kan worden verlaagd.
   Dit proefschrift

6. De prevalentie van perifeer vaatlijden bij familiaire hypercholesterolemie is veel hoger dan klinische verschijnselen doen vermoeden.
   Dit proefschrift

7. Hypercholesterolemie induceert en cholesterolverlaging herstelt de endotheeldysfunctie van macroscopisch normale bloedvaten.
   Stroes et al, Lancet 1995;346:467-71

8. In het licht van de relatie tussen *Helicobacter pylori* en peptische ulcera verdient de associatie van atherosclerose met *Chlamydia pneumoniae* infecties en de aanwezigheid van dit microorganisme in plaques nader onderzoek.
   Kuo et al, PNAS USA 1995;92: 6911-4

9. Ondanks de negatieve publicaties ten aanzien van de behandeling van hypertensie met calciumantagonisten is er op dit moment geen enkele reden om de behandeling te herzien.
   van Zwieten et al, Ned Tijdschr Geneeskd 1995;139:2715-21

10. Elke patiënt met een recent ontstane reumatoïde artritis moet verwezen worden naar een reumatoloog voor agressieve ziektemodulerende behandeling.

11. Absence of evidence is not evidence of absence.
    Altman & Bland, BMJ 1995;311:485

12. Voor veel betawetenschappers geldt maar al te vaak dat ze betwetenschapper zijn.

13. De Nederlandse taal is wetenschappelijk gezien een dialect.

14. 'Bekkeninstabiliteit' t.g.v. toegenomen laxiteit van de ligamenten in de zwangerschap kan een zeer invaliderende aandoening zijn die langdurige, intensieve revalidatie vereist.
    Eigen waarneming

15. Elke academisch werkende specialist wordt geacht evenredig te participeren in kliniek, onderwijs en wetenschappelijk onderzoek. Hierdoor wordt geen optimaal gebruik gemaakt van ieders specifieke kwaliteiten.